CONTENTS

Vol 81 A Fasc. 1 1973

PRINTED IN DENMARK VALD PEDERSENS BOGTRYKKERI A S COPENHAGEN

ACTA PATHOLOGICA ET [illegible]

CON[illegible]

PRINTED IN DENMARK VALD PEDERSENS [illegible]

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style, failure to do so may lead to delay in publication.

Instructions to authors are available on request to the Editorial Board Manuscripts should be submitted to the national editor in their final form as top pages, not carbon copies, in double-spaced type-script in English, French, or German. All written matter, illustrations and references should be submitted at the same time.

Ordinary articles should generally not exceed 8 printed pages and not more than 5 pages of illustrative material. They must contain a summary in English not exceeding 250 words. *Brief reports* for immediate publication must not exceed 1½–2 printed pages Such reports will be published as soon as possible after receipt. Manuscripts will be reviewed by appropriate experts. Since manuscripts will not be insured against loss or damage, contributors are expected to retain duplicate copies of all material submitted for publication Only illustrations of reasonable technical standards will be accepted If the limit of 8 pages is exceeded and, if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication Reference to litterature should conform to the standards of *World Medical Periodicals* or *World List of Scientific Periodicals* In general, reviews of a topic unsupported by original observations will not be accepted.

The official abbreviation is Acta path. microbiol scand. Sect. *A* or *B*

Supplements The publication of supplements will be governed by special rules which can be obtained from the Editor

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section A Pathology

CONTENTS

Vol. 81 A Fasc 2 1973

PRINTED IN DENMARK VALD PEDERSENS BOGTRYKKERI A S COPENHAGEN

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style, failure to do so may lead to delay in publication.

ences should be submitted at the same time

generally not exceed 8 printed pages and not more than 5 pages of summary in English not exceeding 250 words. *Brief* ot exceed 1½–2 printed pages. Such reports will be t. Manuscripts will be reviewed by appropriate experts Since manuscripts will not be insured against loss or damage, contributors are expected to retain duplicate copies of all material submitted for publication Only illustrations of *reasonable technical standards will be accepted* If the limit of 8 pages is exceeded and if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication Reference to litterature should conform to the standards of *World Medical Periodicals* or *World List of Scientific Periodicals* In general, reviews of a topic unsupported by original observations will not be accepted

The official abbreviation is Acta path microbiol scand Sect *A* or *B*

Supplements The publication of supplements will be governed by special rules which can be obtained from the Editor

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Section A Pathology

CONTENTS

Vol 81 A. Fasc 3 1973

Continued on page 3 of cover

PRINTED IN DENMARK VALD PEDERSENS BOGTRYKKERI A S COPENHAGEN

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style, failure to do so may lead to delay in publication.

Instructions to authors are available on request to the Editorial Board Manuscripts should be submitted to the national editor in their final form as *top pages*, not carbon copies in double-spaced type script in English, French, or German. All written matter, illustrations and references should be submitted at the same time

Ordinary articles should generally not exceed 8 printed pages and not more than 5 pages of [illegible] English not exceeding 250 words *Brief* [illegible] 2 printed pages. Such reports will be [illegible] will be reviewed by appropriate ex[illegible] *or damage, contributors are expected* [illegible] or publication. Only illustrations of [illegible] limit of 8 pages is exceeded and, if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication Reference to litterature should conform to the standards of *World Medical Periodicals* or *World List of Scientific Periodicals* In general, reviews of a topic unsupported by original observations will not be accepted

The official abbreviation is Acta path microbiol scand. Sect. *A* or *B*

Supplements The publication of supplements will be governed by special rules which can be obtained from the Editor

CONTENTS

Continued from page 4 of cover

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section A Pathology

CONTENTS

Vol 81 A. Fasc. 4 1973

Continued on page 3 of cover

PRINTED IN DENMARK VALD PEDERSENS BOGTRYKKERI A S

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style, failure to do so may lead to delay in publication.

[illegible] Board Manuscripts should be [illegible] not carbon copies, in double- [illegible] matter, illustrations and refer- [illegible]

Ordinary articles should generally not exceed 8 printed pages and not more than 5 pages of illustrative material They must contain a summary in English not exceeding 250 words *Brief reports* for immediate publication must not exceed 1½–2 printed pages. Such reports will be published as soon as possible after receipt Manuscripts will be reviewed by appropriate experts Since manuscripts will not be insured against loss or damage, contributors are expected to retain duplicate copies of all material submitted for publication. Only illustrations of reasonable technical standards will be accepted If the limit of 8 pages is exceeded and, if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication. Reference to literature should conform to the standards of *World Medical Periodicals* or *World List of Scientific Periodicals* In general, reviews of a topic unsupported by original observations will not be accepted

The official abbreviation is Acta path microbiol scand. Sect. *A* or *B*

Supplements The publication of supplements will be governed by special rules which can be obtained from the Editor

CONTENTS

Continued from page 4 of cover

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Section A. Pathology

CONTENTS

Vol 81 A. Fasc. 5 1973

PRINTED IN DENMARK VALD PEDERSENS BOGTRYKKERI A-S COPENHAGEN

ADVICE TO AUTHORS

Authors must note and adopt the ACTA's customary arrangement and style, failure to do so may lead to delay in publication.

[illegible]

Ordinary articles should generally not exceed 8 printed pages and not more than 5 pages of [illegible] English not exceeding 250 words. *Brief* [illegible] printed pages. Such reports will be [illegible] ill be reviewed by appropriate ex [illegible] damage, contributors are expected [illegible] publication. Only illustrations of [illegible] mit of 8 pages is exceeded and, if corrections in the proof are particularly numerous or the tabular and illustrative material unusually excessive and/or expensive, authors will be requested to contribute to the cost of publication Reference to literature should conform to the standards of *World Medical Periodicals* or *World List of Scientific Periodicals* In general, reviews of a topic unsupported by original observations will not be accepted.

The official abbreviation is Acta path. microbiol. scand Sect. *A* or *B*

Supplements The publication of supplements will be governed by special rules which can be obtained from the Editor

Acta path microbiol scand Section A 81, 1-5, 1973

THYMOMA AND HODGKIN'S DISEASE OF THE THYMUS

JUHA NICKELS, KAARLE FRANSSILA and LARS HJELT

The Central Laboratory of Pathology, University of Helsinki, Helsinki, Finland

Fourteen epithelial thymomas and eight granulomatous thymomas were studied in order to clarify the clinicopathological relationship of these diseases The age distribution, histological picture and clinical course of granulomatous thymoma were quite different from those of epithelial thymoma but similar to those of the nodular sclerosing type of Hodgkin's disease Granulomatous thymoma should therefore be distinguished from epithelial thymoma and perhaps called Hodgkin's disease of the thymus

The nodular sclerosing type of Hodgkin's disease often primarily involves the mediastinum (*Lukes et al* 1966) This causes diagnostic problems in differentiating thymoma from Hodgkin's disease

Ewing (1916) described a special tumour of the thymus which he called granulomatous thymoma Since then it has been regarded as a histological subtype of thymomas in many histological classifications (*e g Lattes* 1962) Because granulomatous thymoma has many similarities with Hodgkin's disease the term for and the classification of the tumour has been criticized (*Legg & Brady* 1965) *Katz & Lattes* (1969) like *Fechner* (1969) suggested that this neoplasm should be considered a peculiar manifestation of Hodgkin's disease of the thymus

The purpose of the present study was to clarify the relationship between the granulomatous thymoma the ordinary epithelial thymoma and Hodgkin's disease

---

Received 16 iii 72 Accepted 21 vi.72

Requests for reprints should be addressed to *Juha Nickels* MD, Patologian keskuslaboratorio, Haartmaninkatu 3, 00290 Helsinki 29, Finland

MATERIAL AND METHODS

The material was collected from the files of the Finnish Cancer Registry from the years 1953 1966 and the files of the Departments of Pathology, University of Helsinki from the years 1957-1970 All the specimens from cases primarily diagnosed as thymomas, either epithelial or granulomatous, were histologically re examined and reclassified without knowledge of any clinical data Only the cases regarded at re examination as epithelial or so called granulomatous thymoma were included in the study They totalled 22 The histological criteria used were those presented by *Lattes* (1962)

Among the epithelial thymomata were included tumours composed of varying mixtures of lymphocytes and epithelial cells The latter have larger nuclei than the former, cytoplasm is clear and rather abundant and they bear close resemblance to reticulum cells The tumour is typically lobulated, the lobules are separated from each other by fibrous septae The epithelial thymomas were divided into four subgroups according to *Lattes* (1962) 1) In the predominantly lymphoid type, lymphocytes are the main component and the epithelial cells are few and scattered. 2) The predominantly epithelial type consists mainly of cells with epithelial appearance The lymphocytes are few 3) The predominantly spindle cell type consists mainly of spindle cells appearing in whorls and fascicles These cells have oval shaped vesicular nuclei and they are usually regarded as epithelial. The number of lymphocytes varies in this type 4) In the 'pseudorosette or "rosette" type, clear

granulomatous thymoma differ in several aspects. In our study, epithelial *thymoma* occurred in all age groups but granulomatous thymoma only in young adults. *Metastases* were common in granulomatous thymoma but were absent in epithelial thymomas. The *prognosis* in cases of epithelial thymoma seemed also to be better. Similar results have been reported earlier (*Katz & Lattes* 1969, *Fechner* 1969). Granulomatous thymoma responds favourably to roentgen *therapy* whereas epithelial thymoma is radioresistant (*Lattes* 1962, *Katz & Lattes* 1969).

No reports of myasthenia gravis associated with the granulomatous thymoma are available, as apposed to the four types of epithelial thymomas. According to *Hirst & Robertson* (1967), approximately 5 *per cent* of all patients with thymoma can be expected to have erythroid hypoplasia. To our knowledge only one case has been reported in connection with granulomatous thymoma (*Remigio* 1971).

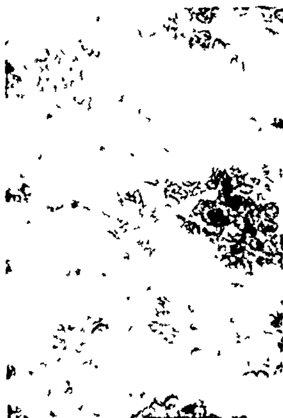


Fig 3 Predominantly rosette type of thymoma. Haematoxylin and eosin × 650

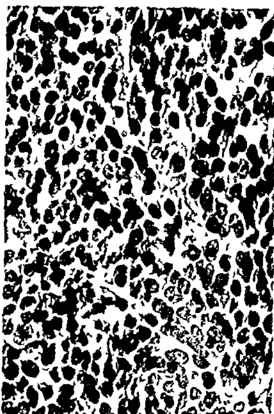


Fig 4 Predominantly epithelial type of thymoma. Haematoxylin and eosin × 650

Histologically, several features of epithelial and granulomatous thymoma are found to differ. Cellular pleomorphism and mitoses are scanty in epithelial thymoma. In granulomatous thymoma however mitoses are often found and the giant cells may be pleomorphic. The eosinophils, plasma cells and neutrophils usually found in granulomatous thymoma are absent in epithelial thymoma.

The granulomatous thymoma resembles the nodular sclerosing type of Hodgkin's disease not only histologically but also in other respects such as age and sex distribution and prognosis (e. g. *Franssila et al* 1967, *Keller et al* 1968).

According to *Lukes et al* (1966), the nodular sclerosing type comprises over 90 per cent of the Hodgkin's disease found in the mediastinum. On the other hand *Franssila*



Fig 5 Granulomatous thymoma mult nucleated tumour cells Hassell's corpuscles and epithelial tissue Haematoxylin and van Gieson × 300

et al (1967) emphasize that the nodular sclerosing type possibly is an entity of Hodgkin's disease with features and characteristics different from the other known types in for example age and sex distribution involvement of the mediastinum and the skeletal system and the presence of typical variants of Reed Sternberg cell There is convincing evidence that the granulomatous thymoma and the nodular sclerosing type of Hodgkin's disease are one and the same disease

The present investigation favours the view that 1) granulomatous thymoma and epithelial thymoma are two distinct entities and should be distinguished 2) that granulomatous thymoma is apparently identical to the nodular sclerosing type of Hodgkin's disease and 3) that the term granulomatous thymoma should be rejected and changed to Hodgkin's disease of the thymus

---

This study was supported by a grant from *J K Paasikivi Foundation (J K)*

REFERENCES

1 *Ewing J* The thymus and its tumors—Report of three cases of thymona. Surg Gynec. Obstet. 22 461–472 1916
2 *Fechner E F* Hodgkin's disease of the thymus Cancer *23* 16–23 1969
3 *Franssila K O Kalima T I & Voutilainen A* Histologic classification of Hodgkin's disease Cancer *20* 1594–1601 1967
4 *Hirst E & Robertson T I* The syndrome of thymona and erythroblastopenic anemia Medicine *46* 225–261 1967
5 *Katz A & Lattes R* Granulomatous thymoma or Hodgkin's disease of thymus? Cancer 23 1 15 1969
6 *Keller A R Kaplan H S Lukes R J & Rappaport H* Correlation of histopathology with other prognostic indicators in Hodgkin's disease Cancer 22 487–499 1968
7 *Lattes R* Thymona and other tumors of the thymus Cancer *15* 1224 1260 1962
8 *Legg M A & Brady W J* Pathology and clinical behaviour of thymomas A survey of 51 cases Cancer *18* 1131 1144 1965
9 *Lukes R J Butler J J & Hicks E B* Natural history of Hodgkin's disease as related to its pathologic picture Cancer *19* 317 344 1966
10 *Remigio P A* Granulomatous thymoma associated with erythroid hypoplasia Amer J clin Path *55* 68 72 1971

Acta path microbiol scand Section A 81, 6–8, 1973

ALCIAN BLUE-SILVER IMPREGNATION A METHOD DIFFERENTIATING BETWEEN ACID CARBOHYDRATES, RETICULIN FIBRES AND COLLAGEN FIBRES

HANS LYON and POUL PRENTO

Histochemical Laboratory, Department of Pathological Anatomy, Kommunehospitalet Copenhagen Denmark
(Chiefs Hemming Poulsen Per Christoffersen Jens Christian Andreasen Hans Lyon)

A standardized silver impregnation method coupled with Alcian blue staining is described The method purports to differentiate between acid carbohydrates, reticulin fibres and collagen fibres It is suggested that the method could be used in the study of transition zones between reticulum fibres and collagen fibres

The routine procedure in our laboratory for silver impregnation of reticulin fibres (*Gordon & Sweets* 1936) has given some rather capricious results, especially as regards the differentiation between reticulin and collagen fibres We therefore decided to attempt to standardize our methods

Lillie (1965) points out, that all silver methods for the demonstration of reticulin fibres consist of six major steps, viz oxidation sensitization silver impregnation, reduction, toning, and fixing A study of the significance of the various steps is in progress However when examining the fifth step toning with acid auric chloride, we found such a dramatic change in the appearance of the final section when this step was completely omitted that attention should be brought to this point in a preliminary publication When toning is omitted a precise differentiation between reticulin fibres and collagen fibres is achieved as reticulin fibres are stained black while collagen fibres and acid mucosubstances are stained golden brown A distinction between collagen fibres and acid mucosubstances may further be achieved by counterstaining with a cationic dye such as Alcian blue

Received 30 viii 72 Accepted 19 ix 72

Requests for reprints should be addressed to *Hans Lyon*, Patologisk Anatomisk Institut Kommunehospitalet, Øster Farimagsgade 1399 Copenhagen K, Denmark

MATERIALS AND METHODS

The work has been carried out on sections of liver biopsies and sections from different parts of the gastrointestinal tract from our routine laboratory Most consistent results were obtained with a modification of the procedure originally described by *Gordon and Sweets* (1936)

The modification consists in placing the formaldehyde reduced silver impregnated sections in an Alcian blue solution instead of toning them Our procedure is as follows

1 Bring formalin fixed paraffin sections to water

2. Oxidize for 1½ minutes in acidified perman ganate solution
3. Wash in tap water for 5 minutes and rinse in two changes of distilled water
4. Bleach until white in 1 per cent oxalic acid
5. *Wash in tap water for 5 minutes* and rinse in two changes of distilled water
6. Sensitize for 15 minutes in 2.5 per cent aqueous iron alum ($(NH_4)_2SO_4 \cdot Fe_2(SO_4)_3 \cdot 24 H_2O$)
7. Wash in 3 changes of distilled water for 2 minutes
8. Impregnate in silver diammine hydroxide solu tion until sections are transparant (approx 1 minute)
9. Wash in several changes of distilled water
10. Reduce in 3.6 per cent w/w aqueous solution of formaldehyde (10 per cent formalin) for 30 seconds
11. Wash in tap water for 5 minutes and rinse in two changes of distilled water Microscopic control (If sections are overimpregnated re peat the process from step 6)
12. Stain in 1 per cent solution of Alcian blue 8GX in 0.1 M hydrochloric acid for 20 minutes
13. Rinse in 2 changes of 0.1 hydrochloric acid for 1 minute in each gently moving the sec tions up and down
14. Wash briefly in distilled water
15. *Dehydrate clear and mount in synthetic resin*

Solutions

Acidified permanganate 95 ml 0.5 per cent aqueous potassium permanganate is added to 5 ml 0.3 M (3 per cent) sulphuric acid

Silver diammine hydroxide To 5 ml of 10 per cent aqueous silver nitrate 20 M (34 per cent *w/w) ammonia is added drop by drop until* the precipitate which forms is just dissolved Add 5 ml 0.75 M (3 per cent) sodium hydroxide Add 20 M ammonia drop by drop until the resulting precipi tate dissolves The solution does not clear com pletely Make up to 50 ml with distilled water

RESULTS

Reticulin fibres dark brown to jet black Col lagen fibres golden yellow to golden brown Acid mucosubstances blue to green

DISCUSSION

Rinehart (1930) has *proposed two methods* for the impregnation of connective tissue fibres one involving gold toning, the other not He describes how toning transforms the golden brown reaction of collagen in silver sections into a rose red These *results are* similar to ours, though toning in our hands leads to the collagen becoming grey to purple grey *Gordon and Sweets* (1936) have in their original paper pointed out, that the toning procedure is merely a refinement and may be omitted

Furthermore they note that the impregna tion procedure is reversible as sections may be completely destained in the alum mordanting solution *following the formaldehyde reduction* but preceding toning Slides destained in this manner may then be reimpregnated Destain ing is, however, not possible once the slides have been toned in gold We have been able to confirm these results The significance of this last finding is, that it is possible to more or less standardize by microscopic control the final results when toning is omitted, and in cases of overstaining destain and reimpreg *nate*

We have not in the literature found any reports of combined Alcian blue–silver im pregnation methods

Acid mucosubstances are stained yellowish brown in silver impregnated sections which have not been treated with Alcian blue

The colour obtained is very similar to that exhibited by collagen fibres The problem of differentiating between mucosubstances and collagen fibres may be overcome by counter staining with a cationic dye We have here found Alcian blue suitable

In the combined Alcian blue–silver impreg nated sections no confusion can arise between collagen fibres and mucoproteins and/or acid mucopolysaccharides

CONCLUSION

The combined Alcian blue–silver impregna tion method described here would seem to be of merit in the study of transition zones be tween reticulin fibres and collagen fibres, e g *in beginning cirrhosis of the liver*

Supported in part by *Statens Naturvidenskabelige Forskningsrad*

Miss *Jette Pedersen* and Miss *Jytte Kræmmer Jensen* are sincerely thanked for their unfailing interest in the preparation of the material used in this work just as we are indepted to Mrs *Gerhild Olsen* for careful typewriting of the manuscript

REFERENCES

1 *Gordon H* & *Sweets H H* A Simple Method for the Silver Impregnation of Reticulum Amer J Path *12* 545 551, 1936

2 *Lillie, R D* Histopathologic Technic and Practical Histochemistry 3rd ed McGraw Hill Book Company, New York Toronto Sydney and London 1965 pp 525 536

3 *Rinehart, J F* Reticulum The Occurrence of Reticulum Fibrils in Capillary Endothelium. A New Method of Demonstration II The finer Capillary Bed Amer J Path *6* 525-539, 1930

Acta path microbiol scand Section A 81, 9 15, 1973

ARTEFACTUAL STAINING OF THE PERIPHERAL ZONE OF NEEDLE BIOPSIES

HANS LYON and POUL PRENTØ

Histochemical Laboratory, Department of Pathological Anatomy, Kommunehospitalet, Copenhagen, Denmark
(Chiefs Hemming Poulsen, Per Christoffersen, Jens Christian Andreassen, Hans Lyon)

A description of artefactual staining in 156 needle biopsies of the liver is given The following representative methods were employed Alcian blue—basic fuchsin mixture pH 3, fast green FCF pH 8 azure A—eosin Y mixture pH 5, gallocyanin—chromalum, Feulgen procedure and aluminium—haematein Changes in stainability were seen in the 3–15 most peripheral cell layers and affected both cell nuclei and cytoplasm as well as sinusoidal lining A dissociation of nucleoprotein and denaturation of protein in the outermost layers of the biopsy effected by the needle is suggested as being the cause of the altered stain ability The consequences of these findings for the evaluation of needle biopsies is dis cussed

It has previously (*Scheuer* 1968) been pointed out, that artefactual staining is frequently seen in the outermost cell layers in needle biopsies from the liver Scheuer has illustrated this by Perls' ferrocyanide method for the demonstration of ferric iron In the peripheral parts of needle biopsies a bluish staining is frequently seen of nuclei and cyto plasm in hepatocytes as well as of the sinus oidal lining without any relation to the actual content of ferric iron in the biopsy

In this paper an attempt has been made to find out which staining methods are most prone to give this artefactual staining of the peripheral zone of needle biopsies, and which methods are not The probable underlying mechanism is discussed in detail

Received 30 viii 72 Accepted 19 ix 72

Requests for reprints should be addressed to the authors, Patologisk Anatomisk Institut Kommune hospitalet Øster Farimagsgade 1399 Copen hagen K Denmark

MATERIAL AND METHODS

The material consists of 156 percutaneous liver biopsies performed by the method of Menghini and using needles with a core diameter of either 1 1 or 1 6 mm Of these biopsies 136 are from patients with a histological diagnosis of acute viral hepatitis, 7 from patients with a diagnosis of large bile duct obstruction 3 from patients with a diagnosis of toxic hepatitis and 10 from patients whose liver biopsies on histologic examination were without pathological changes In addition 16 surgical biop sies have been included from patients operated for chronic ulcers of the stomach

The biopsies were received at the Pathological Anatomical Institute at Kommunehospitalet in Copenhagen during the period December 1968 to August 1971 All biopsies were fixed in phosphate buffered 10 per cent formalin pH 6 8 After pre paration of paraffin blocks serial sections were cut on a rotary microtome set at 7 μm

TABLE 1 *A Comparison of the Stainability of Different Structures in the Peripheral Zone and the Central Zone of Needle Biopsies of the Liver*

Tissue *structure*		Procedure						
		Alcian blue basic fuchsin	Fast green FCF	Azure A eosin Y	Gallocyanin chromalum	*Feulgen*	Aluminium haematein	Colloidal iron
Nuclei	Peripheral zone*	*Blue*	*Green*	*Purple*	Bluish black	Magenta	Blue	*Blue*
	Central zone	Red	Unstained	Blue	Bluish black	Magenta	Blue	Unstained
Cytoplasm	Peripheral zone	*Blue* or red	*Green*	Purple	Greyish black	Unstained	Light blue	Unstained
	Central zone	Red	Unstained	Purple or red	Greyish black	Unstained	Light blue	Unstained
Sinusoidal lining	Peripheral zone	*Blue*	Unstained	Unstained	Unstained	Unstained	Light blue	*Blue*
	Central zone	Red	Unstained	Unstained	Unstained	Unstained	Light blue	Unstained
Connective tissue	Peripheral zone	*Blue*	Unstained	*Purple* or red	Unstained	Unstained	Light blue	*Blue*
	Central zone	Red	Unstained	Red	Unstained	Unstained	Light blue	Unstained

* Discrepancies in the staining of structures in the periphery when compared to the same structures in the central zone of the biopsy have been put in italics

The following procedures were employed

Alcian blue 8 GX (E Gurr) 0 25 per cent and basic fuchsin (E Merck) 0 01 per cent in 0 1 M citric acid phosphate buffer pH 3 0 Staining time 20 m (*Prento & Lyon* unpublished results)

Fast green FCF (E Gurr) 0 1 per cent pH 8 0 in 0 01 M HCl/borate without preceding nucleic acid extraction (*Prento & Lyon* 1972a) Staining time 30 m

Azure A (E Gurr) 0 1 per cent and eosin Y (E Gurr) 0 1 per cent in 0 1 M citric acid phos phate buffer pH 5 0 Staining time 60 m (*Lillie* 1965)

Gallocyanin-chromalum according to *Einarson* (1951) Staining time 18 h

Feulgen procedure Purine hydrolysis with 5 N HCl at room temperature for 15 m (*Deitch et al* 1968)

Aluminium Haematein according to Mayer Staining time 10 m

Colloidal iron according to Mowry 60 m in colloidal iron (*Pearse*, modification 1968)

In 14 of the cases part of the needle biopsy was removed before processing to paraffin and 7 μm sections were made on a SLEE cryostat These sections were stained in an identical manner to the paraffin sections All of these 14 biopsies were from patients with acute viral hepatitis

RESULTS

When the Alcian blue basic fuchsin technique, the fast green FCF method or the azure A eosin Y procedure were used a typical peripheral effect was encountered in all of the needle biopsies with a more intense staining or otherwise altered stainability of the structures in the 3–15 most peripheral cell layers Such changes were on the other hand not seen with the gallocyanin chromalum technique, the Feulgen nuclear reaction, or the aluminium haematein technique

The results are summarized in Table 1 With the Alcian blue basic fuchsine method it was seen that not only cytoplasm and the sinusoidal lining were stained blue in the peripheral portions of the biopsies in contrast to the red staining in the central portions of the biopsies but also the nuclei were stained blue with Alcian blue in the peripheral part of the biopsy Further, connective tissue in the periphery of the biopsy was stained blue, whereas it was stained red in the central portions It is of interest, that these changes in the stainability of connective tissue frequently extend farther into the biopsy than the other changes recorded above (Fig 1)

With the fast green FCF method no staining was normally achieved of cytoplasm, nuclei or sinusoidal lining in the central portions of the biopsies In contrast to this the outer 3–15 layers of liver cells in the needle biopsies exhibited a fairly well marked cytoplasm, and a fainter, but distinct, colouring of the majority of nuclei The connective tissue was unstained both in the central and in the peripheral parts of the biopsies

In azure A eosin Y stained sections an increased stainability with both azure A and eosin Y of nuclei, cytoplasm and connective tissue was observed in the peripheral parts of the needle biopsies

In the sections stained by the gallocyanin-chromalum method the Feulgen procedure, and the aluminium haematein technique no changes in peripheral stainability were found in the needle biopsies

With colloidal iron staining of nuclei, sinusoidal lining and connective tissue was observed in the peripheral zone, while all these structures were unstained in the central zones of the biopsies

In none of the surgical biopsies was any peripheral staining effect observed

In all the cryostat sections results were identical to those obtained with paraffin sections from the same needle biopsies

DISCUSSION

Individual Reactions

For reasons of clarity the results of the individual reactions are first discussed separately before attempting to give a common explanation for the findings

Alcian blue basic fuchsin These are two cationic dyes Under the conditions applied here the majority of phosphate groups and non carbohydrate carboxylate groups are inaccessible for Alcian blue either on account of blockade or for steric reasons (*Prento &*


A
B
C
D

Lyon unpublished results) If, however, protein is removed by enzymatic extraction, the phosphate groups in nucleic acids react strongly and preferentially with Alcian blue in this method (*Prento & Lyon*, unpublished results)

Fast green FCF This is an anionic dye which stains positively charged amino groups in proteins At the pH employed (pH 8) the only charged amino groups are to be found in basic proteins These are the globins in haemoglobin and myoglobin and the histones found in nucleoproteins Whereas the former react freely and are intensely stained with fast green FCF, the latter are not stained, as they are blocked by the negative charges of nucleic acids If a preliminary extraction of nucleic acids is performed with 1 N hydrochloric acid 60° C or with trichloroacetic acid (*Alfert & Geschwind* 1953), the staining capacity of the histones is, however, revealed

Azure A eosin Y Azure A is a cationic dye very often used as a nuclear stain because of its ability to demonstrate nucleic acids Eosin Y is an anionic dye behaving essentially as fast green FCF

Gallocyanin chromalum This dye is, when used at pH 1 6, selective for nucleic acids, and it is relatively insensitive to blocking (*Prento & Lyon* 1972b)

Feulgen procedure This reaction is specific for the demonstration of DNA and is independent of the degree of protein blockade

Mayer's aluminium haematein This metal—dye complex stains nuclei intensely

Several macromolecules may be involved, e g nucleic acids and histones

Colloidal iron This is not a staining method in the strict sense of the word, but is a method based on the affinity of positively charged metal colloids to negatively charged tissue groups These groups will at the conditions applied be identical to those described above under Alcian blue

Interpretation

The increased stainability of the nuclei and cytoplasm of cells in the peripheral parts of the needle biopsies with anionic dyes, with Alcian blue and with colloidal iron must, in accordance with the above, be due to a dissociation of nucleoprotein complexes This dissociation must take place when the biopsy is removed, as also frozen sections of the biopsies exhibit changes in peripheral stainability, whereas surgical biopsies fixed in the same manner as the needle biopsies do not show any changes in peripheral stainability However, this does not explain the fact, that also the sinusoidal lining and the connective tissue exhibit an increased staining intensity Here, it is near to hand to compare the results with the different staining results obtained according to the fixative employed It is a well known fact, that formaldehyde fixation enhances staining with cationic dyes or perhaps more correctly primarily enhances staining with both cationic and anionic dyes and secondarily inhibits staining with anionic dyes to a greater or lesser degree by the formation of azomethine bondings (*Baker* 1966) On the other hand coagulative fixatives such as ethanol and acetic acid in Clarke s fixative favour the anionic dyes as there is no blocking of the amino groups and the changes induced in the tertiary structure of the proteins make both carboxylate and amino groups more readily accessible

It would seem probable, that in the case of needle biopsies the preliminary effect is a denaturation of proteins in the peripheral parts of the biopsy (pressure? tension? heat?) This would cause a dissociation of

Fig 1 Alcian blue basic fuchsin 0 25 per cent/0 001 per cent pH 4 staining of a liver biopsy For reasons of photographic contrast the concentration of basic fuchsin was reduced from the normal 0 01 per cent to 0 001 per cent

A Peripheral zone (250 ×)

B Sinusoidal lining (400 ×)

C Portal area with connective tissue (400 ×)

D Nuclei of hepatocytes and Kupffer cells (400 ×)

nucleoproteins and in opening up of the tertiary structure of the proteins Both these changes would increase stainability with both anionic and cationic dyes If the biopsy is then formaldehyde fixed azomethine bondings will easily be established between the amino groups but the relative acidophilia of the tissue will still be increased when compared with the central areas of the biopsy The increased basophilia will of course still be present following formaldehyde fixation

Even though the changes described above thus chiefly seem to affect protein and protein complexes, changes of other biopolymers cannot be excluded In fact glycogen seems to be more consistently present in the peripheral parts of the needle biopsies than in the central parts as exhibited by a more intense PAS reaction in the periphery of the biopsy Further enzymes being proteins must be expected to reflect similar changes

The diffuse artefactual staining seen in the peripheral zone of needle biopsies with Perls ferrocyanide reaction already referred to in the introduction to this paper could possibly be explained by a mechanism similar to that given for Alcian blue and colloidal iron with formation of colloidal iron complexes to nucleic acids and proteins

The results seen with routine haematoxylin eosin with increased acidophilia in the peripheral zone are in accordance with the above This also applies to the more or less pronounced increase in staining with acid fuchsin in the van Gieson picrofuchsin procedure, where increased permeability of the proteins clearly plays a part

The above explanation of the mechanism inducing the peripheral effect is supported by other well known observations Changes of the cells identical to the biopsy induced may be seen when the tissue has been pinched by forceps or other instruments or may be seen if the tissue has been torn by a defect in the microtome knife On the other hand indirect support may be obtained from the fact, that the surgical biopsies in this material, where special care was taken to avoid squeezing the biopsies did not exhibit any changes in stainability in their peripheral portions

It must therefore be recommended that in the assessment of liver biopsies any morphological findings *only* observed in the peripheral layers of the biopsies should be regarded with utmost caution

Of the three methods used in this investigation for demonstrating the artefact, the Alcian blue basic fuchsin procedure gives the demarcation of the peripheral artefactual staining most distinctly, and this method may therefore be recommended for assessment of how large the peripheral zone is It should be noted that in the first few sections and in the last sections from a block containing a needle biopsy the peripheral zone may well include the whole section

It should further be noted, that the changes may extend much further into the biopsy by transmission through the non elastic connective tissue This is a fact to be borne in mind especially when assessing needle biopsies containing much connective tissue as in cirrhosis

Finally these findings indicate that when material is to be obtained for electron microscopic examination this should be taken from the central portion of the needle biopsy and not from the extremities or peripheral portions of the biopsy

---

Supported in part by *Statens Naturvidenskabelige Forskningsråd*

Miss *Jette Pedersen* and Miss *Birgitte Bigom* are sincerely thanked for their unfailing interest in the preparation of the material used in this work

REFERENCES

Alfert M & Geschwind I I A selective staining method for the basic protein of cell nuclei Proc Nat Acad Sci *39* 991 999 *1953*

Baker J R Cytological technique 5th ed Science Paperbacks and Methuen & Co Ltd London 1966 pp 39–44

Deitch A D Wagner D & Richart R M Conditions influencing the intensity of the Feulgen reaction J Histochem Cytochem *16* 371 379 1968

Einarson, L. On the theory of gallocyanin chromalum staining and its application for quantitative estimation of basophilia. A selective staining of exquisite progressivity. Acta Path Microbiol Scand 28 82–102, 1951

Lillie, R D Histopathologic technic and practical histochemistry 3rd ed, Mc Graw—Hill Book Company, New York 1965, pp 162–165

Prento P & Lyon H Unpublished work

Prento P & Lyon, H Nucleoprotein staining. An analysis of a highly standardized trichloroacetic acid—fast green FCF procedure. Histochem J (in press)

Prento, P & Lyon, H Nuclear staining. A comparitive study. Histochem J (in press)

Scheuer, P J Liver biopsy interpretation. Ballière, Tindall & Cassell ltd, London 1968, pp 1–3

Acta path microbiol scand Section A 81, 16–20, 1973

THE HISTOLOGICAL APPEARANCE OF HYPERFUNCTIONING THYROIDS FOLLOWING VARIOUS PRE-OPERATIVE TREATMENTS

P. C. Eggen and R. Seljelid

Department of Pathology,
the Norwegian Radium Hospital, Oslo, Norway
Head: R. Eker

It is well known that the pre-operative treatment of thyrotoxic patients influences the histology of surgical specimens. However, a description of the exact relationship between the type of pre-operative treatment and the histology is not available. The aim of the present investigation was to obtain some information on this point. It was found that the histology of thyrotoxic glands varied considerably and that the 'classical' picture was seen only in about one third of the cases. Thyroids from patients treated only with iodine showed more variation within the specimen, taller cells, larger nuclei and nucleoli, more marginal vacuolization, and more germinal centres, but less vascularization of the stroma than thyroids from patients treated with sulphur-containing thyrostatica.

During histological examination of surgical specimens from patients with thyrotoxicosis, it became apparent that the histology in the same clinical situation was very variable. Also, observations made during routine diagnostic work suggested that the type of pre-operative treatment of the patients would influence the histological pattern. This influence of medication on thyroid histology is commented in most text-books (*1, 2, 4, 6*). However, there is little exact information on the relationship. The aim of the present investigation was to contribute some information on this point. The questions we asked were the following:

1. How do different pre-operative treatments influence the histological appearance in surgical preparations from thyrotoxic glands?
2. Are there, in spite of variation, still some features common to all specimens in thyrotoxicosis, and are these features sufficiently clear to allow diagnosis?

MATERIAL AND METHODS

The Department of Pathology, the Norwegian Radium Hospital receives a large number of surgical specimens from numerous hospitals throughout the country. The hospitals referred to in this investigation routinely send all their surgical material to this laboratory for examination.

We started out by collecting all preparations with the diagnosis thyrotoxicosis sent to us during the years 1969 and 1970. Toxic adenomas and nodular goiters were excluded, leaving only the preparations with diffuse changes. Since we would like to go into some detail with respect to the pre-operative treatment, we choose to concentrate on specimens from 5 large hospitals (Bærum sykehus, Gjøvik sykehus, Namdal sykehus, Tromsø sykehus and Tønsberg sykehus). Fifty-seven specimens were studied, 38 from women, 20 to 63 years of age (mean 39.2), and 19 from men, 16 to 63 years of age (mean 37.6). Two to 6 paraffin sec-

Received 28.vi.72. Accepted 21.ix.72.

TABLE 1 *Average Score for the Three Types of Treatment*

Preoperative treatment	Group 1 Sulphur cont. thyro-statics & thyroxin	Group 2 Sulphur cont thyro statics & iodine	Group 3 Iodine
Follicle size	0.8	0.9	0.8
Follicle size variation	0.9	1.1	1.5
Follicle shape	0.8	0.7	0.9
Follicle cell height	1.3	1.3	1.6
Nuclear size	1.1	1.2	1.6
Nucleolar size	1.1	1.2	1.6
Nuclear size variation	0.9	0.9	1.2
Colloid stainability	0.8	0.7	0.6
Variation in stainability	0.9	0.5	0.7
Marginal vacuoliza tion of follicles	1.1	1.1	1.4
Colloid macrophages	0.2	0.3	0.6
Stroma amount	1.2	1.2	1.1
Collagen amount in stroma	1.1	1.4	1.2
Vascularity of the stroma	0.8	1.1	0.5
Lymphocytes in stroma	0.7	0.7	0.8
Plasma cells in stroma	0.6	0.6	0.7
Macrophages in stroma	0.5	0.5	0.6
Distribution of in flammatory cells	0.9	0.9	1.6
Histological pattern	0.5	0.5	1.3

tions from each specimen were examined after staining with haematoxylin and eosin

To enable a reasonable objective comparison of the specimens a semi-quantitative scoring scheme of the relevant histological features was developed (Table 1) Considering the fact that histological evaluation is of necessity subjective and must be more unreliable the more complex the scoring system is we choose a simple system allowing only 3 ratings 0–1 2 (3) The histological features were scored in the following way

Referring to size 0 1 2 indicated small, medium or large and to the amount of various cells or characteristics absence presence or abundantly present Regarding the shape of the follicles the three groups signified round both round and irregular, or predominantly irregular The staining of the colloid was either faint, moderate or intense, the distribution of inflammatory cells either diffuse, focal or focal with formation of germinal centres The histological pattern as a whole was grouped as 0 if the appearance was monotonous throughout the specimen, as 1 if there was a slight, and as 2 if there was extreme variation

Information on the pre-operative treatment was obtained from the files of the 5 hospitals and was not known to the authors at the time of the histological examination

The 57 patients could be grouped as follows

- Group 1 24 patients given sulphur-containing thyrostatics plus thyroxin
- Group 2 21 patients treated with sulphur-containing thyrostatics plus iodine (Lugol's solution)
- Group 3 12 patients treated only with iodine (Lugol's solution)

It was not possible to obtain exact information on the duration of treatment in individual cases However the overall trend was that iodine (Lugol's solution) was administred for 1–2 weeks, whereas the sulphur-containing drugs as well as thyroxine were given for months or years.

For each histological feature, the average score for the three types of treatment was calculated and compared It must be understood that these numbers do not express degrees of histological changes, but rather the relative frequencies of the predominant scores

RESULTS

Some of the histological features did not show any clear-cut relationship to the preoperative treatment (Table 1) This was e g the case with the follicle shape, colloid stainability, amount of stroma, inflammatory cells in the stroma In some respects, however, specimens from the three groups of treatment differed consistently These histological features are displayed in Fig 1 It is seen that preparations from patients treated only with iodine showed much more variation in the histological structure, with some lymphocyte infiltration and a tendency towards distinct germinal centres in the lympoid tissue (Fig 2) Also the follicles varied more in size in the preparations from the iodine group, the follicle cells were taller, and the nuclei larger with more distinct nucleoli and also some variation in the size of the nuclei (Fig 3)

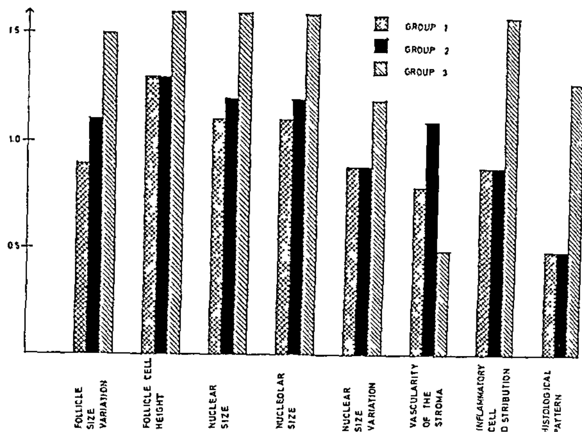


Fig 1 Histogram illustrating relative frequencies of histological features in the three groups of different pre operative treatment rated 0 1 2 (See Material and Methods)

There was more marginal vacuolization in the iodine group and more 'colloid macro phages'

Specimens from the two other groups showed a more uniform picture in all re spects The vascularization of the stroma was pronounced in these two groups and most evident in specimens from patients treated with sulphur containing thyrostatics plus iodine

DISCUSSION

Textbook description of the characteristic histological features in hyperfunctioning glands (1 2 4 6) includes tall epithelial cells small follicles a decreased amount of colloid which is thin pale and vacuolated around the edges Accumulation of lympho cytes, often forming distinct reaction centres is described as a consistent feature This de scription of the histology of hyper functioning thyroids is not substantiated wholly by the present study On the contrary the histo logical appearance varies considerably In our series only 17 cases (~ 30 per cent) corre sponded in all respects to the description of the classical hyperthyroid gland as mentioned above, and in 8 cases (14 per cent) no signs typical for the hyper functioning gland were found

We have not been able to establish any new criteria that would be of diagnostic value in all cases Thus we are left with a high percentage of cases (~ 70 per cent) where only some of the typical signs were present and where diagnosis would be dif ficult to establish on the basis of histology alone

Turning to the individual features in the classical description it appears from our results that increased cell height may be the most reliable criterium for hyper functioning

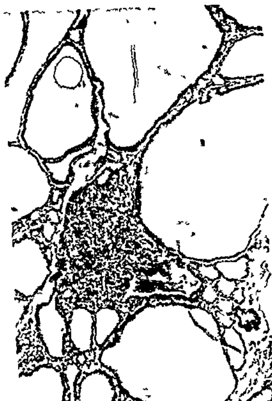


Fig 2 Thyroid from patient treated with iodine only There is considerable variation in the shape and size of the follicles A large accumulation of lymphocytes is seen × 120



Fig 3 Thyroid from patient treated with iodine only The follicle cells are tall, the nuclei large and a little irregular The colloid stains faintly and there is prominent marginal vacuolization × 300

of the gland, being present in about 50 per cent of the cases This is true for all groups

There was no obvious decrease in the average follicle diameter in any group However, in glands treated with iodine alone there was a larger variation in follicle diameter than in the other groups

The colloid stained less intensely than normal in all of the groups However, this is a very difficult criterion on which to base a diagnosis, since the staining of the colloid will vary considerably for technical reasons Nor is peripheral vacuolization of the colloid a reliable diagnostic criterion, because it is an artefact caused by fixation and therefore will vary in an unpredictable way according to the handling of the tissue prior to histological examination (8)

Infiltration of the stroma with lymphoid cells, was found in 40 of our 57 cases (70 per cent), and germinal centres in 20 of our cases (35 per cent) This latter histological feature varied considerably in our three treatment groups In groups 1 and 2, germinal centres were found in 33 per cent and 24 per cent, and in group 3, including patients treated only with iodine germinal centres were found in 67 per cent

The differences in histological structure caused by the three types of treatment reflect obviously the difference in action of the various drugs on the cellular level It is known that the main effect of the sulphur-containing thyrostatics is a blocking of the organification of iodine during thyroglobulin synthesis (4, 5)

The mode of action of iodine is less well understood The short duration of treatment, usually about 10 days (5, 7), may account for the variation in the histological picture

The sulphur containing thyrostatica had been supplemented with either thyroxin or iodine in the pre operative procedure in groups 1 and 2 The effect of thyroxin is probably mediated by way of the feed back-mechanism via the pituitary gland, and the result was a definite decrease in vascularization of the stroma as compared with the group treated with sulphur containing thyrostatica plus iodine

In this context it is also interesting to note the variation and relative increase in nuclear size as well as the larger nucleoli found in the iodine group These features are often found in patients with increased amounts of circulating TSH e g in inborn metabolic disorders of the thyroid (9)

REFERENCES

1 *Anderson W A D* Pathology 5 ed Vol 2 Mosby Company St Louis 1966 p 1082 1084

2 *Curran R C* Farbatlas der Histopathologie Springer Verlag Berlin Heidelberg & New York 1966 p 37

3 *Godal T Ådresen R & Seljelid R* Hypothyreose etter strumektomi ved non toksisk struma T norske Legeforening *90* 1690–1692 1970

4 *Harrison T R* Principles of internal medicine 5 ed McGraw Hill Book Company, New York Toronto Sydney & London 1966 p 421–423

5 *Kuschinsky G & Lullmann H* Pharmakologie 3 ed Georg Thieme Verlag Stuttgart 1967 p 183 186

6 *Lamberg B A* Sköldkörtlens sjukdomar 2 ed Remedia fennica Helsingfors 1969 p 138 254

7 *Laake K* Farmakoterapi Johan Grundt Tanum Forlag Oslo 1972 p 111 113

8 *Seljelid R* Thyroid lysosomes in health and disease In Trump B & Arstilla A (Eds) Pathology of cellular membranes In press Academic Press New York & London 1972

9 *Seljelid R* Unpublished work

Acta path microbiol scand Section A 81, 21-36 1973

COURSE OF ALLOXAN DIABETES IN DUCT-LIGATED RATS

A Functional and Morphological Study

CURT EDSTRÖM

The Institute of Pathology (Head Prof S Falkmer), University of Umeå, S 901 87 Umeå 6 Sweden

The course of alloxan diabetes during 19 months was studied in 62 duct ligated and 26 sham-operated female rats Nine additional rats were used as buffer injected sham operated controls. Alloxan was administered 5-6 weeks after duct ligation The mortality among the alloxan treated rats was high and many animals died during ether anaesthesia at glucose tolerance tests or from late sequelae of alloxan induced kidney lesions In duct ligated rats both the hypoglycaemic phase 4-8 hours after alloxan administration and the subsequent hyperglycaemia during the first 4 days were distinctly marked After these days during which signs of instability were observed the fasting blood glucose level in the duct ligated rats returned within the normal range after 5 months at which stage the only 3 surviving sham-operated controls still were hyperglycaemic The latter showed, how ever varying degrees of alloxan induced kidney lesions The pancreas was studied by light microscopy in 29 duct ligated and in the 3 surviving sham-operated alloxan-diabetic rats From the first week and onwards signs of regenerative phenomena of the islet tissue, con sisting of large clear sparsely granulated cells of the so-called "agranular type, were ob served in the islets of the alloxan-treated animals Furthermore, some evidence was ob tained that the large sparsely granulated cells had the ability to differentiate into β-cells Despite this normalization of the fasting blood glucose level of the alloxan-diabetic duct ligated rats and despite these morphological signs of β cell regeneration, no alloxan treated animals showed a complete recovery from their diabetes as glucose tolerance tests performed 8 months after alloxan administration were still of diabetic type However histological examination of the pancreas in 2 rats 19 months after alloxan administration were by light microscopy found to present mainly normal islet cells in both the atrophied and the intact parts of the parenchyma and there was no glucosuria or hyperglycaemia

Preceding studies of the interrelation between duct ligation and alloxan diabetes in rats showed that duct ligation did not interfere with the sensitivity of alloxan induced dia betes (*Edström* 1971 b) When the duct liga tion was performed after administration of alloxan restoration of the structure and function of the damaged endocrine pancreas was observed to be more rapid in duct ligated rats than in sham-operated controls (*Zuceens* & *Bouman* 1967, 1969) These signs of high regenerative capacity conform with the fact that both light microscopical (*Ed ström* & *Falkmer* 1967, 1968, *Zuceens* & *Bouman* 1967, 1969) and ultrastructural (*Bo-*

Received 3 viii 72 Accepted 3 viii 72

Requests for reprints should be addressed to Dr Curt Edström Institute of Pathology, University of Umeå S 901 87 Umeå 6 Sweden

quist & *Edström* 1970) signs of islet neoformation in the atrophied pancreatic parenchyma obtained after duct ligation have been described

In order to obtain some further information of this allegedly increased regenerative capacity of duct-ligated pancreatic parenchyma after alloxan administration, it was thought worth while to extend the preceding alloxan experiments (*Edström* 1971 b, *Edström* & *Hassler* 1971) to comprise also a long term study of the course of alloxan diabetes evoked in duct ligated rats These rats were given alloxan 5–6 weeks after duct ligation and studied functionally by analyses of the fasting blood glucose level and by glucose tolerance tests, as well as structurally in the light microscope and in the transmission electron microscope The results of the latter part of the study are given in a separate report (*Edström* & *Boquist* 1973)

By using sham-operated controls, and due to the presence of intact pancreatic parenchyma in subtotally duct ligated rats (*Edström* 1971 c), it was also hoped to obtain some information about the regenerative capacity of the islet parenchyma of the normal rat after alloxan administration This problem has previously been studied and isolated reports have just appeared (*Lazarow* 1952), it is of practical importance as alloxan diabetic rats are commonly used in several types of long term experiments, *e g* in studies on various types of diabetic microangiopathy and kidney lesions (*Orskov et al* 1965, *Jensen* & *Lundbaek* 1968, *Vargas et al* 1970)

MATERIAL AND METHODS

The basic experimental procedures were the same as in the preceding reports (*Edstrom* & *Falkmer* 1967, 1968, *Edstrom* 1971 a b c) In all 97 non diabetic female Sprague Dawley rats approximately 2 months old and weighing about 200 g at the start of the experiments were used The presence of glucosuria proteinuria and haematuria was studied by means of Haemocombistix® (Ames Co) paper strips and the blood glucose assays were made by the glucose oxidase procedure (*cf Boquist* 1967) Sixty two rats were duct ligated and the remaining 35 animals were sham-operated The animals were used for the alloxan experiments 5 6 weeks after the operation Before the alloxan administration all rats were controlled by blood glucose determination and urine analysis to prove that they were free from obvious hyperglycaemia and glucosuria (*cf Okamoto* & *Yamamoto* 1954)

A survey of the various types of experimental procedures used in the present study is given in Table 1 Fifty-one duct ligated rats weighing about 240 g, and 26 sham-operated controls weighing about 210 g received a single diabetogenic dose of alloxan intraperitoneally *ca* 105 ± 10 mg/kg body weight (*Edstrom* 1971 b) The remaining 9 sham-operated rats (weighing 186 g ± 2*) received plain buffer in doses of corresponding volumes intraperitoneally The animals had always free access to food and water except for 0–8 hours after alloxan administration

* The standard error of the mean (S E M)

TABLE 1 *Survey of the Distribution of the 97 Expe*

Kind of operation	Total number of rats	Fasting blood glucose level 0–4 days	Fasting blood glucose level and glucose tolerance tests 1 week–8 months	Intraperitoneal administratior							
				Hours				Light microscopica Weeks			
				¼	1	24	48	1	2	4	5
Duct ligation	62	16	25	1	1	1	1	1	1	1	1
Sham operation	35	14	12								

* These rats were used for both blood glucose assays and light microscopical examination

Sixteen duct ligated rats weighing 255 g ± 3* and 14 sham operated controls weighing 215 g ± 5*, of the alloxan treated animals mentioned above were used for blood glucose investigations during the first hours and days after administration of alloxan The blood glucose assays were alternated at random between the rats to avoid exsanguination Single samples from at least 8 rats were taken on each occasion of observation The animals had always free access to food and water except for 0 8 hours after injection After the investigation all the rats were killed except two which were allowed to live until 19 months after the administration of alloxan

Fasting blood glucose was assayed in 25 duct ligated rats weighing 228 g ± 3* and in 12 sham operated controls weighing 196 g ± 2* at intervals varying between 0 and 8 months Single samples from at least 2 rats were taken at each observation time except for the initial duplicate samples from the duct ligated animals The rats were fasted over night Glucose tolerance tests were performed on the same animals during approximately the same intervals by the same technique as previously (*Edstrom* 1971 a), using 3 g glucose/kg body weight It applies also in this case that at least 2 rats were glucose loaded at each observation time (Figs 3 and 4) The 9 buffer treated rats were handled in a similar way and used as controls The exact number of animals investigated at each observation time is given in Fig 1 The fate of these rats is given in Table 2

The remaining 10 duct ligated rats were used for morphological studies only They were killed 15 60 minutes 1 2 days and 1, 2, 5 weeks as well as 1 and 3 months after the alloxan administration Eight rats from the blood glucose assay experiments were also used for light microscopical examination 3, 5, 8, and 19 months after alloxan administration

The remaining alloxan untreated 11 duct ligated rats, weighing 235 g ± 4*, received 40–50 mg alloxan/kg body weight (*Lukens* 1948 *Schmidt* 1967) injected into the caval vein and were killed 15, 30 minutes, 1, 24 48 hours and 1 week after the injection

Specimens from the pancreas from these last mentioned 29 rats and from 3 sham-operated rats sacrificed 5 months after alloxan administration, were exposed to light microscopical examination and further stained with chrome haematoxylin ponceau fuchsin (*Hultquist* 1962), van Gieson's stain, and with silver impregnations according to *Grimelius* (1969) and *Hellerstrom & Hellman* (1960) Furthermore by way of a pilot study, specimens from liver kidneys and myocardium were taken from 3 duct ligated rats killed or dying 3 and 5 months after alloxan administration and from the 3 alloxan treated sham-operated controls sacrificed at 5 months The paraffin sections were stained with haematoxylin-eosin and/or van Gieson's stain Sections from the kidneys were also stained with the PAS reagent and with Best's carmine for detecting glycogen

* The standard error of the mean (S E M)

RESULTS

Mortality and Body Weight Fluctuations

The number of alloxan treated rats decreased gradually during this investigation because of death to occur during ether anaesthesia at glucose tolerance tests or from sequelae of alloxan induced kidney lesions (Table 2) It is likely that also the 3 sham

Rats Used in the Present Study of Various Kinds of ...ments

Alloxan (105 ± 10 mg/kg) examination of pancreas					Intravenous administration (50 ± 10 mg/kg)						Buffer
											Intraperitoneal administration of buffer
Months					Hours					Days	Fasting blood glucose and glucose tolerance tests
)	3	5	8	19	¼	½	1	24	48	7	
1	2§	1* 3*	4*	2*	2	2	2	1	2	2	9

§ One of these two rats was used for both blood glucose assays and light microscopical examination

TABLE 2 *Blood Glucose Values of Duct Ligated and Sham Operated Rats Dying or Kille*

		Days 3	4	5
Duct ligated	Dying spontaneously	681 (2)	447 (2)	
	Dying at blood sampling			
	Killed			
Sham operated	Dying spontaneously			523 (2) 648 (2) § §
	Dying at blood sampling			
	Killed			

Numbers within brackets indicate the time (in days weeks, or months respectively) when the last blood sample was taken

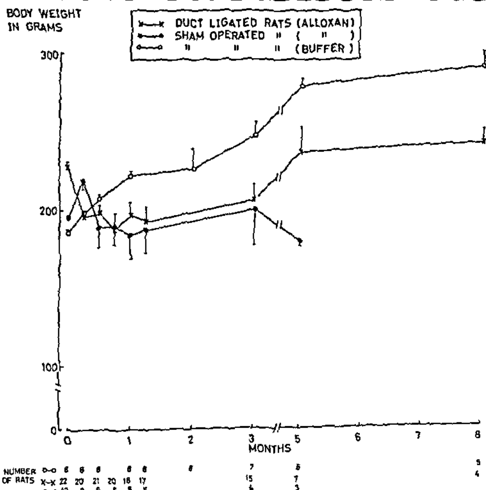


Fig 1 Mean body weights of duct ligated and sham operated rats at various time intervals following administration of alloxan or plain buffer The vertical bars indicate ± standard errors of the means (S E M)

before or at the End of the Predetermined Observation Time Intervals after Alloxan Administration

Weeks 1	3	4	5	Months 2	3	5	8
273	63 (2)	66 (3), 70	82 (4), 440, 440		354, 429	59, 66 (3)*, 99 (3), 160*, 285, 423, 360, 417	
273					105 (2)		
339	230			80, 125 (5 weeks)			74 (5), 67*, 96*, 86*, 97*
					407*, 410*	617*	

* These animals were also used for morphological examination

§ Lost blood samples

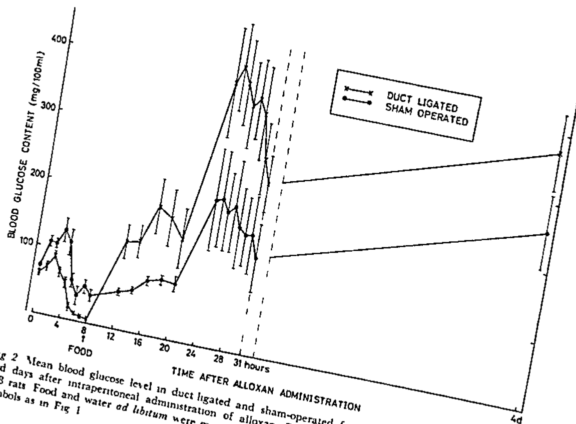


Fig 2 Mean blood glucose level in duct ligated and sham-operated female rats during the first hours and days after intraperitoneal administration of alloxan Each value is based on single samples from 5–8 rats Food and water *ad libitum* were given 8 hours after administration of alloxan Same signs and symbols as in Fig 1

operated rats surviving for 5 months after alloxan administration very soon might have died spontaneously if they had been allowed to live after the last glucose tolerance test

The changes in the mean body weights at various observation times of the rats in the experimental group and of those in the two control groups are given in Fig 1 The curve showing the mean fluctuations in the body weight of the alloxan diabetic sham operated rats lends further support to the assumption that these rats would have died spontaneously soon after 5 months if they had not been sacrificed 5 months after alloxan administration

Fig 1 also shows that the alloxan administration evoked a transient decrease in the mean body weight, lasting for a couple of months both in duct ligated and sham operated rats However in the duct ligated rats surviving for 3 months after alloxan administration the average gain in body weight seemed to be the same as that in buffer injected sham operated controls This lends additional support to the hypothesis that these surviving rats were recovering from their alloxan diabetes The gradually decreasing number of rats implied that only a few rats were available for functional and structural analysis after the longest periods of observation Hence these results should be taken with the greatest reservation before any conclusions are drawn

Initial Blood Glucose Variations in Fed Rats

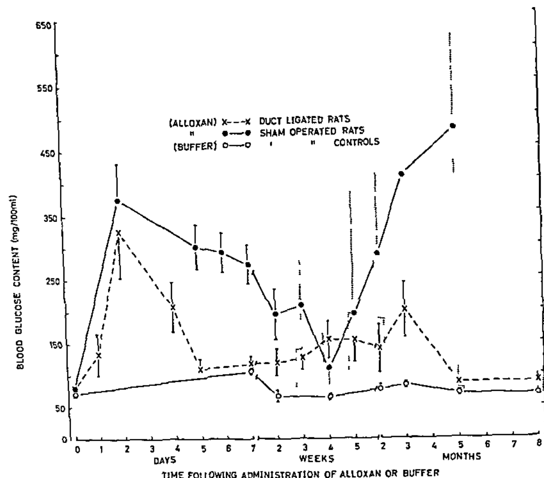
The mean level of blood glucose in duct ligated and sham operated rats after administration of a single diabetogenic dose of alloxan at various brief time intervals is given in Fig 2 It applies to both groups of animals that the characteristic well known triphasic curve with a rapid initial hyperglycaemic phase was obtained followed by a deep hypoglycaemia 5 8 hours after the injection and the ultimate lasting hyperglycaemia The hypoglycaemic phase was particularly marked in the duct ligated rats two of these were seized with severe hypoglycaemic muscular convulsions and had to be rescued by oral administration of glucose None of the sham operated animals showed such marked hypoglycaemic symptoms Also in the third phase about 24 hours after the alloxan administration, the duct ligated animals showed a more marked reaction than the sham operated ones However these differences were not significant As also mentioned in the next paragraph the controls only treated with plain buffer did not show any marked variations in the blood glucose level during the first 3 hours or during the subsequent 8 months

Long Term Fasting Blood Glucose Levels

The mean long term level of blood glucose in alloxan treated duct ligated and sham operated rats fasted over night is given in Fig 3 It applies to both types of experimental animals that the blood glucose level had its peak approximately 2 days after alloxan administration upon which a decrease was observed The decrease seemed to proceed at a higher rate in the duct ligated rats in which the blood glucose level was approximately normal 5 days after alloxan administration In contrast the blood glucose level in the sham operated rats showed a transient decrease after 4 weeks followed by a new marked increase terminating in a severe diabetic state of the animals 5 months after the alloxan administration The duct ligated rats remained almost normoglycaemic until 3–4 weeks after alloxan administration Then a slow increase lasting for up to 3 months after alloxan administration occurred it was followed by a return to the normal range during the periods of observation of 5 8 and 19 months after alloxan administration The sham operated controls injected with plain buffer only were normoglycaemic at all observation times

Glucosuria

Random samples taken on these various occasions of observation showed that the occurrence of glucosuria proteinuria and haematuria conformed well to the observations made in the preceding alloxan experi



NUMBER OF RATS															
x---	20	20	8	18	20	–	20	20	19	16	16	16	13	5	4
●—	12	–	9	–	8	8	8	6	5	4	5	4	2	3	–
o—	8	–	–	–	–	–	8	8	–	8	–	8	7	6	5

Fig 3 Mean fasting blood glucose level in duct ligated and sham-operated alloxan treated rats as well as in control rats treated with plain buffer Blood glucose assays were made in 2–20 rats at each of the various time intervals after injection Only the initial samples were duplicates The number of rats used at the various time intervals is given below the diagram The interrupted vertical bars indicate the range of the individual values Otherwise, same signs and symbols as in Fig 1

ments (*Edström* 1971 b, *Edström* & *Hassler* 1971) No systematic studies were made, however

Glucose Tolerance Tests

The glucose tolerance tests in all the alloxan treated animals—both duct ligated and sham-operated—were of the diabetic type at all observation times (Fig 4) The mean values of fasting blood glucose level before the glucose tolerance tests, and the values 5 hours thereafter, were lower in the duct ligated rats than in the alloxan-treated sham operated ones The sham operated rats treated with plain buffer only, showed normal glucose tolerance (Fig 5)

Thus, despite the normalization during long periods of observation of the fasting blood glucose level of the duct-ligated rats, their glucose tolerance curves were still of diabetic type, and did not differ significantly from those of the sham-operated controls

TABLE 3 *Survey of the Light Microscopical Changes Observed in the Islet Parenchyma of Duct-Ligated Rats at Various Time Intervals after Alloxan Administration*

Time interval after alloxan administration	Pancreatic islets in the proximal "intact" portion				Pancreatic islets in the distal atrophied portion			
	α_1-cells	α_2 cells	β-cells	"Agranular" cells	α_1-cells	α_2-cells	β-cells	"Agranular" cells
0 2 days	normal	normal	necrotic remnants	rare	rare	rare	necrotic remnants	rare
1 week	normal	normal	absent	frequent	rare	rare	absent	frequent
2 weeks	frequent	frequent	rare	increased frequency	normal	normal	rare	increased frequency
1–5 months	frequent	frequent	rare	frequent	frequent	frequent	rare	frequent
8 months	normal	normal	almost normal	frequent	normal	normal	almost normal	frequent
19 months	normal	normal	normal	rare	normal	normal	mostly normal	rare

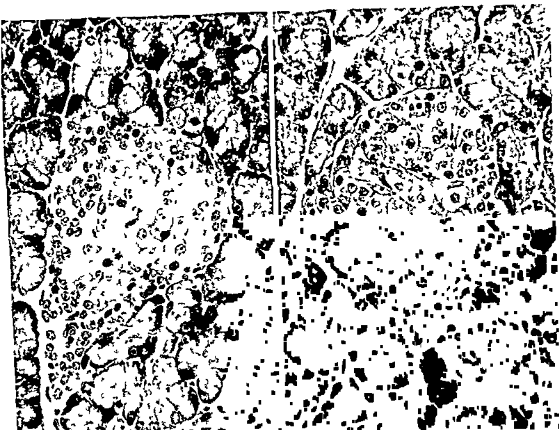
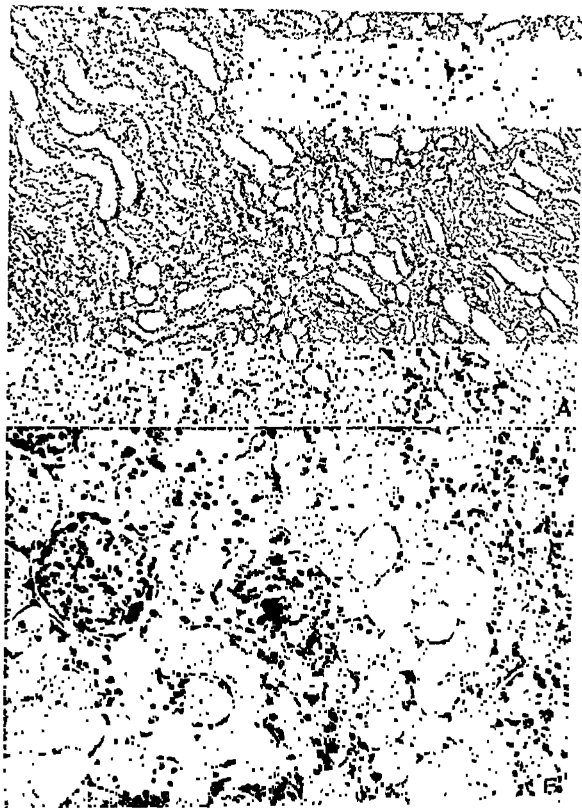


Fig 6 Medium power photomicrographs showing the islet parenchyma of an alloxan diabetic duct ligated rat 8 months after alloxan administration (A) and that after 19 months (B) The islet parenchyma of the latter animal is almost normal but some large pale but clearly granulated cells still occur in the central part In the islets of the former animal numerous α cells are seen and large pale sparsely granulated cells are situated in the central part These latter cells are supposed to be young immature forms of regenerating β cells The photomicrographs are from the intact part of the ligated pancreas Bouin s fixative Chrome haematoxylin ponceau fuchsin × 480

differences between the histopathological pictures in the severely diabetic sham operated controls killed at this observation time and in the normoglycaemic duct ligated animals killed at the same time interval

Exocrine Pancreas The acinar pancreatic parenchyma of the 'intact' portion proximal to the ligatures did not show any alloxan-induced lesions at any time of observation, and any necroses in the remnants of ducts or in the tubular structures of the atrophied portion were not in evidence The regenerative phenomena observed in the latter are to be described in the subsequent report (*Edström & Boquist* 1973) The exocrine pancreas of the alloxan diabetic sham operated controls was normal

Kidney In one duct ligated rat which died spontaneously 3 months after alloxan administration, marked degenerative lesions were found in the epithelium in both the proximal and distal convoluted tubules Marked parenchymatous degeneration with atrophy of tubular cells and secondary cystic dilatations of the lumina of the kidney tubules were also observed In the medulla, the tubular epithelium showed hydropic degeneration with large amounts of glycogen There was no obvious pyelonephritis or pyelitis The lesions were so severe and wide spread in this rat



А
Б

that renal failure was considered to be the main cause of its death The kidneys of the other two duct ligated rats studied 3 and 5 months after alloxan administration were essentially normal Thus, any signs of diabetic glomerulosclerosis were not observed

The 3 sham operated, severely alloxan diabetic rats, sacrificed 5 months after alloxan administration, showed areas of "alloxan nephrosis" of the same kind as described above including wide spread parenchymatous degeneration of the epithelium of both the proximal and distal convoluted tubules and marked glycogen infiltration in other parts of the tubuli as well as secondary cystic dilatations (Figs 7 A and B) There was, however, no diabetic glomerulosclerosis

Liver and Myocardium In the few cases studied 3–5 months after alloxan administration, the histopathological picture was normal in both liver and myocardium Thus, no differences between duct ligated and sham operated animals were observed

DISCUSSION

It appears from the results that the duct ligated animals showed signs of greater sensitivity to alloxan during the first hours during which the hypoglycaemic values were lower and the hyperglycaemic values higher than those of the sham operated rats The underlying mechanism in this trend to greater initial response to alloxan of rats duct ligated 6 weeks previousloy is not clear The hypoglycaemia may be due to smaller deposits of available glycogen, as the carbohydrate metabolism and the digestion in duct ligated rats has been reported to be disturbed (*Okamoto & Yamamoto* 1951) After these first days of observation, the duct-ligated rats seemed to sustain the diabetogenic action of alloxan better than the sham operated controls Whether or not this statement is true, however, cannot be deduced from the data of the present study because the 3 sham operated alloxan-diabetic control rats showed severe kidney lesions The differences observed might be due to a higher capacity of the pancreatic islets in the duct ligated rats for regenerating β cells as proposed by *Zuœens & Bouman* (1967, 1969) and observed in human beings with cystic fibrosis of the pancreas (*Brown & Madge* 1971), but might as well reflect merely the higher frequency of "alloxan nephrosis" in the sham operated rats Both interpretations seem quite plausible In retrospect, it can be said that it would have been more profitable if the experimental procedure used by *Ørskov et al* (1965) had been applied for the administration of alloxan by which the kidneys are protected from the initial damage by the necrotizing agent However, this was not done and the tubules showed about the same changes as those previously described by *Vargas et al* (1970) It can be speculated whether these kidney lesions, detected 3–5 months after alloxan administration, represent only remnants of an initial alloxan damage or whether they are essentially the result of the diabetic state of the animal The diabetes may also play a secondary role by interfering with normal tubular regeneration (*Vargas et al* 1970) Any relevant explanations of this problem are not offered by the present experimental results It is interesting to note, however, that any diabetic glomerulosclerosis did not appear 3 5 months after alloxan administration This is in accord with other experimental results obtained in alloxan diabetic rats (*Ørskov et al* 1965) where observation times up to 8–12 months are needed in order to obtain diabetic glomerulosclerosis

Fig 7 Low (A) and medium power (B) photomicrographs of the kidney of a hyperglycaemic glucosuric sham operated rat 5 months after administration of alloxan There is a sponge like appearance of both the cortex and the medulla due to atrophy of the tubular epithelium and secondary cystic change of the lumina (A) The epithelium of the proximal and distal convoluted tubules show severe parenchymatous degeneration with loss of nuclei and a marked swelling of the cytoplasm almost occluding the lumina Other parts of the tubules show marked hydropic degeneration with large amounts of glycogen in the cytoplasm (Fig 7 B centre and right) The glomeruli are normal Bouin's fixative PAS reaction × 70 (A), × 360 (B)

No obvious histopathological changes were seen in the liver and the heart of the few animals studied However, in rats with long-term diabetes with a high hyperglycaemia, submicroscopic changes of the myocardial cells have been noted previously by *Lebkova & Bondarenko* (1969) and may thus contribute to the high frequency of deaths among the diabetic rats

Although the results of the present experiments do not allow any conclusions concerning the course of alloxan diabetes in the normal rat, it is clear that the regeneration from alloxan diabetes of duct ligated rats differs significantly from that of another alloxan sensitive rodent, *viz* the Chinese hamster (*Boquist* 1968) Whereas the latter species showed normalization of the fasting blood glucose level within 8–9 days and a return to the normal of the glucose tolerance 4–5 weeks after alloxan administration, the rats were still hyperglycaemic 3 months after alloxan administration and a diabetic glucose tolerance test persisted even up to 8 months This poor regenerative capacity of rat pancreatic islet parenchyma was also observed by *Lazarow* (1952) Some of his alloxan diabetic rats did not show any spontaneous improvement of their diabetic state until 12–20 months after alloxan administration These peculiar differences in the regenerative capacity of the pancreatic islets in a murine rodent species with known hereditary trait for endogenous diabetes and in another murine rodent species with no such traits are difficult to account for Whether structural differences between regenerating islets of the rat and the Chinese hamster are in existence, will be the subject of the subsequent report (*Edström & Boquist* 1973) The appearance of these large pale sparsely granulated cells in the central β cell region of the rat islets during the phase of expected regeneration reminds in the light microscope closely of the regenerative pattern observed in the Chinese hamster (*Boquist* 1968) So far, the most obvious difference seems thus to be temporal However, it is difficult by light microscopy to decide with certainty whether or not large, clear, sparsely granulated cells in the β cell region just represent young immature forms of insulin producing cells Some of these cells might equally well be degranulated mature β cells Some aspects of this possibility can be deduced from an ultra structural investigation of these large clear cells which is to be the main theme of the subsequent report (*Edström & Boquist* 1973)

On the assumption that the results of *Lazarow's* (1952) experiments on non operated rats are comparable with the present data on the course of alloxan diabetes in duct ligated rats, it seems as if the regenerative capacity of the parenchyma after duct ligation actually is superior to that of normal rat pancreas, as mentioned in the Introduction However, comparisons of this kind are hardly permissible, because too many factors may influence the results of experiments of this type

---

This work was supported by grants from the *Medical Faculty of Umeå* the *Swedish Diabetes Association* and the *Swedish Medical Research Council* (Project No B72 12X 718 07A)

REFERENCES

Boquist L Morphology of the pancreatic islets of the non diabetic adult Chinese hamster *Cricetulus griseus* Light microscopical findings Acta Soc Med Upsal *72* 331 344 1967

Boquist L Alloxan administration in the Chinese hamster I Blood glucose variations glucose tolerance and light microscopical changes in pancreatic islets and other tissues Virchows Arch Abt B Zellpath *1* 157 168 1968

Boquist L & Edstrom C Ultrastructure of pancreatic acinar and islet parenchyma in rats at various intervals after duct ligation Virchows Arch Abt A Path Anat *349* 69–79 1970

Brown R E & Madge G E Cystic fibrosis and nesidioblastosis Arch Path *92* 53 57 1971

Edstrom C Glucose tolerance of rats at various intervals following ligation of the pancreatic ducts Acta Soc Med Upsal *76* 39–48 1971 a

Edstrom C Alloxan sensitivity of rats at various intervals following ligation of the pancreatic ducts Acta Soc Med Upsal *76* 77–86 1971 b

Edstrom C Further quantitative structural studies of the pancreatic islet parenchyma in rats

with duct ligation Acta Soc Med Upsal *76* 127-138, 1971 c
Edstrom, C & *Boquist L* Alloxan diabetes in duct ligated rats Light and electron microscopic findings Acta path microbiol scand *81A* 47-56, 1973
Edstrom, C & *Falkmer, S* Qualitative and quantitative morphology of rat pancreatic islet tissue five weeks after ligation of the pancreatic ducts Acta Soc Med Upsal *72* 376-390, 1967
Edström, C & *Falkmer, S* Pancreatic morphology and blood glucose level in rats at various intervals after duct ligation. Virchows Arch Abt A Path Anat *345* 139-153, 1968
Edstrom, C & *Hassler, O* Functional capacity, alloxan sensitivity and vascular supply of pancreatic islet parenchyma during the first week after duct ligation An experimental study in rats with microangiography Acta Soc Med Upsal 76 211 222, 1971
Grimelius, L Studies of adult human pancreatic islet cells with a new silver nitrate stain Abstr Upsal Diss Med 62 1 23, 1969
Hellerstrom, C & *Hellman, B* Some aspects of silver impregnation of the islets of Langerhans in the rat Acta Endocr *35* 518-532 1960
Hultquist, G T Nuclear size in the cells of Langerhans islets during starvation in rat Acta Anat (Basel) *49* 281-287, 1962
Jensen, V A & *Lundbaek, K* Fluorescence angiography of the iris in recent and long term diabetes Diabetologia *4* 161-163 1968
Lazarow, A Spontaneous recovery from alloxan diabetes in the rat Diabetes *1* 363-371, *1952*
Lebkova, N P & *Bondarenko, M F* The ultrastructure of myocardial cells in experimental alloxan diabetes in rats Kardiologija *9* 121-124, 1969
Lukens, F D W Alloxan diabetes Physiol Rev 28 304-330, 1948
Okamoto, K & *Yamamoto, T* Experimental studies on the production of diabetes by the ligation of the pancreatic duct of rabbits Kobe J Med Sci *1*. 15-30, 1954
Orskov, H , Steen Olsen, T , Nielsen, K , Rafaelsen, O J & *Lundbaek, K* Kidney lesions in rats with severe long term alloxan diabetes I Influence of age, alloxan damage, and insulin administration Diabetologia *1* 172-179, 1965
Schmidt, R Der Alloxandiabetes Morphologie, Chemismus und Literatur Nova Acta Leopoldina 32 1-749, 1967
Vargas, L , Friederici, H H R & *Maibenco, H C* Cortical sponge kidneys induced in rats by alloxan Diabetes *19* 33-44, 1970
Zweens, J & *Bouman, P R* Neoformation of insulin producing islets following ligation of the pancreatic ducts in normal and alloxan diabetic rats Acta Physiol Pharmacol Neerl *14* 529-530, 1967
Zweens, J & *Bouman, P R* Neoformation of insulin producing islets following ligation of the pancreatic ducts in normal and alloxan-diabetic rats Diabetologia *5* 60, 1969

Acta path microbiol scand Section A 81, 36-46 1973

PERIRENAL FIBROSIS AS A CAUSE OF UREMIA AND HYPERTENSION IN HUMAN TRANSPLANTED KIDNEYS

P FAARUP, B HENRIKSEN, K OLGAARD and J J DAHLAGER

The University Institute of Pathology and the Nephrological Dept P Rigshospitalet and the Institute of Pathology, Bispebjerg Hospital

In 3 renal transplanted patients perirenal fibrosis developed within one year after transplantation The kidney function slowly decreased and uremia and hypertension developed in spite of intensive immunosuppressive treatment The characteristic morphological changes in kidneys with perirenal fibrosis were atrophy and fibrosis localized in the superficial cortex At biopsy this condition could easily be separated from immunologic and infective lesions of the parenchyma as well as from vascular complications causing decrease in kidney function and hypertension The clinical histories indicate that the perirenal fibrosis was caused by complicating perinefritis and/or perirenal hemorrhage in all three cases In rats a closely similar lesion could be experimentally produced It is concluded that perirenal fibrosis should be considered as an alternative diagnosis to chronic rejection and vascular complications in transplanted patients with late graft failure and hypertension

Rejection, vascular complications, and pyelonephritis are commonly found in renal transplants In the present work a new complication is described This consists of perirenal fibrosis evolving parallel to the appearance of uremia and hypertension

MATERIAL AND METHODS

A Human Material

In the 3 patients investigated the following techniques were applied

1) *Morphological methods* The renal biopsies and kidneys were fixed in formalin as well as in Helly s fluid In addition to the routine stains the sections were stained by a silver stain and PAS stained according to *Faarup & Petri* (1969)

The kidney volume was found by the principle of *Archimedes* and the cortial volume of the kidney was found by planimetry of 1 cm thick serial sections of the kidneys as previously described (*Hegedus & Faarup* 1972 in press)

2) *Renography technique* Isotope renography was performed using a conventional technique with a scintillation counter I^{131} labelled hippuran was given rapidly as a bolus intravenously in a dose of 0 3 uCi per kg body weight

With the well hydrated patient in a supine position the location of the transplant was found by palpation and the detector placed at a constant distance over the kidney The information was analysed by a channel analyzer and recorded continously for twenty minutes by a graphic recorder

The renogram marked N on Figs 2 8 and 12 represents renograms from transplated patients with a creatinine clearance of more than 80 ml/min and normal blood pressure These measurements were done one or more years after transplantation

A renogram consists normally of three segments *The first segment corresponds to the sudden upstroke* of the tracing and represents the vascular phase *The second segment* termed the secretory phase is characterized by a slower ascent which reaches a peak *In the third segment* the tracing

Received 6 vii 72 Accepted 12 viii 72

Requests for reprints Dr *Poul Faarup* The University Institute of Pathology Juliane Maries vej 2100 Copenhagen

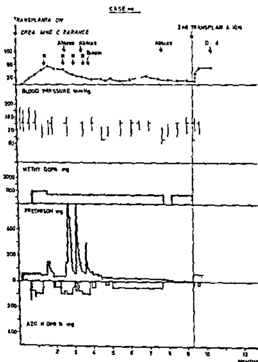


Fig 1 Diagram showing creatinine clearance blood pressure and antihypertensive as well as immunosuppressive treatment in case no 1 The rejection crisis clinically suggested perirenal abscesses and time of biopsy are indicated by arrows The kidney function is found to be slowly decreasing during which there is a gradual rise in blood pressure irrespective of the immunosuppressive treatment (*cf* Figs 7 and 11)

declines gradually This is the so-called excretory phase

B Experimental Model

As an experimental parallel to the human kidneys investigated unilateral perirenal fibrosis was induced in 4 rats in which the left kidney was inclosed in gauze containing talcum powder according to the technique described by *Page* (1939) 6–10 months later the kidneys were removed fixed in neutral formalin and stained as previously described

CASE REPORTS

Case no I 30 year old man with terminal uremia and severe hypertension in whom a rapidly progressive glomerulonephritis was present

Kidney transplantation was done with a necrokidney with one possible major HLA incompatibility At the same time bilateral nephrectomy was performed Prior to the transplantation the patient was normotensive

Due to an initial ischemic damage of the transplant the creatinine clearance slowly increased up to approximately 70 ml/min 1½ month after the transplantation At the same time the blood pressure increased to a level of about 160/105 From this time anti hypertensive treatment was instituted (Fig 1)

The steroid dose was temporarely increased at 1½ 2 and 3 months after the transplantation due to decreasing kidney function The diminishing kidney function and the increased blood pressure were clinically interpreted as rejection *However no effect was found despite the large steroid doses applied*

2½ months after the transplantation a perirenal abscess surrounding the transplanted kidney was diagnosed and approximately 200 ml of pus were drained This abscess reappeared 4 and 8 months after the transplantation The kidney function slowly deteriorated Thus the creatinine clearance was declining from 70 ml/min 1½ months after the transplantation to 10 ml/min 8 months after the transplantation Bacteriuria was persistently found

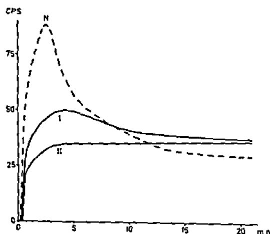


Fig 2 Case no 1 N Renogram used as a reference in transplanted kidneys as seen after one year without complications

Curve I Renogram 1½ month after transplantation Moderately good uptake of hippuran maximum peak with normal time and fairly good phase of elimination

Curve II Renogram 8 months after transplantation Severely diminished uptake and continuous accumulation of hippuran within 20 min

The renograms demonstrate a progressive decrease in blood flow and a progressive depletion of the tubular uptake of hippuran indicating a severe functional decrease of the tubules



Fig 3 Case no 1 Biopsy of the renal cortex from the first transplanted kidney, in which perirenal fibrosis was present (the capsule is not shown in the figure) In the superficial part of the cortex (A), significant atrophy and fibrosis are present No inflammatory changes are found and most glomeruli are vascularized, although atrophic (*cf* Fig 4)

In the deeper part of the cortex (B), only small and focally placed atrophic parenchymal changes are present (*cf* Fig 5)

The arrow in the lower left corner in this and the following figures points radially out to the surface of the kidney (PAS stain × 70)

A second kidney transplantation was performed 9 months after the first transplantation with a necro kidney with one possible major HLA incompatibility Due to peroperative bleeding during the second transplantation removal of the first transplanted kidney was not done Now creatinine clearance increased rapidly to about 55 ml/min and the blood pressure remained normal without antihypertensive treatment (Fig 1)

Five weeks later the patient died from a severe hepatitis and acute pancreatitis

Isotope renography was made at intervals of 2 4 days after the transplantation The first renogram showed a well defined secretory phase and the curve continued to ascend during the 20 minutes the investigation lasted which is common in cases of tubular ischemic damage of the kidney

The renogram was almost normalized 6 weeks after the transplantation (Fig 2) From that time onwards a gradual deterioration occurred A diminished secretory phase delayed peak and slow *excretory* phase were seen Three months after the transplantation no indication of an excretory phase was present in the curve which ascended slowly during the whole test period

Kidney morphology A surgical biopsy of the kidney cortex was made 4 months after the first transplantation At operation the surgeon observed a thick fibrous capsule surrounding the kidney and firmly attached to the parenchyma In the biopsy significant atrophy and interstitial fibrosis were found in the superficial part of the cortex (Figs 3 and 4) Deeper in the parenchyma only insignificant degenerative changes were present No inflammatory changes were observed and no signs of immunological complications could be found In the atrophic superficial part of the renal cortex no epithelioid cell hyperplasia was present in the juxtaglomerular apparatus (Fig 4) although this was regularly the case deeper in the parenchyma (*Fig 5*)

At autopsy the presence of a thick fibrotic capsule around the first renal transplant was confirmed (Fig 6 a) The greatest *thickness of the* capsule was seen on the ventral side of the kidney the diameter being up to 6 mm as opposed *to the*

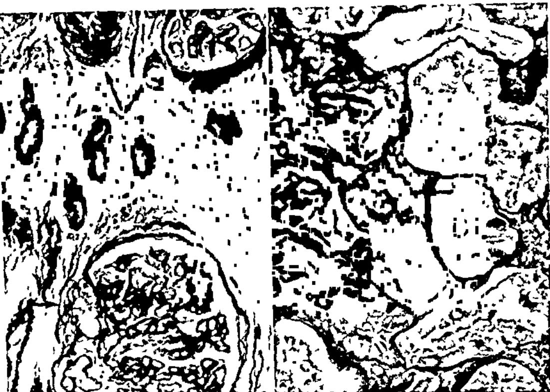


Fig 4 Case no 1 In the superficial cortical tissue (A zone in Fig 3), a few epithelioid cells are found in the vascular wall of the afferent arteriole close to the glomerulus (arrow) No epithelioid cells are seen in the interlobular artery or in the arteriole in the upper part of the figure (arrows) (PAS stain, × 875)

Fig 5 Case no 1 In the deeper part of the renal cortex (B in Fig 3) many epithelioid cells are found in the wall of the afferent arteriole (a) near the glomerulus (arrows) Juxtaglomerular granules are not identified in the cells DT Distal tubule (PAS stain, × 875)

normal thickness of the capsule of the second transplanted kidney (Fig 6 b) Close to the thickened capsule of the first transplant severe fibrotic changes were focally present, and on the ventral side of the kidney an abscess cavity was found of a size about 100 ml

The renal morphology of the first transplant was identical to that of the biopsy In the second transplant no significant parenchymal changes were present The size of the first transplanted kidney was somewhat smaller than that of the second the kidney volumes being 150 and 200 ml each The total cortical volume of the kidneys were 67 ml and 116 ml respectively

As stated above the cause of dead in this patient was severe hepatitis followed by acute pronounced pancreatitis

Case no 2 31 year old man with terminal uremia and hypertension in whom chronic glomerulonephritis was found

Transplantation was done with a kidney from the brother of the patient without demonstrable major incompatibility At the same time a bilateral nephrectomy was done

The postoperative course was complicated by uroplania due to a leakage at the uretero pelvic anastomosis and by repeated infections The creatinine clearance was initially 70 ml/min and the blood pressure about 140/80 In the next year the blood pressure was continously normal and creatinine clearance was between 65 and 100 ml/min The only immunosuppressive treatment applied in this period was azothioprine, 100 mg per day (Fig 7)

One year following the transplantation, rejection was clinically suspected due to declining creatinine clearance and increasing blood pressure *In spite of temporary treatment with large steroid doses, no effect was obtained* (Fig 7)

22 months following the transplantation the creatinine clearance was further decreased to 24 ml/min and the blood pressure now was 200/130

At intravenous urography, reduced absorbtion and elimination without obstruction or stenosis of pelvis or ureter were found Renal arteriography showed vessels of normal caliber without stenosis or other vascular abnormalities in the transplant

Isotope renography was at first made 22 months after the transplantation A good secretory phase with a delayed maximum and a poor excretory phase were recorded (Fig 8, curve 1) The slope indicates the presence of a diminished renal blood flow in a kidney with a normal functioning tubular mass

The renogram was repeated 31 months after the transplantation and at this time showed identical features apart from poorer uptake

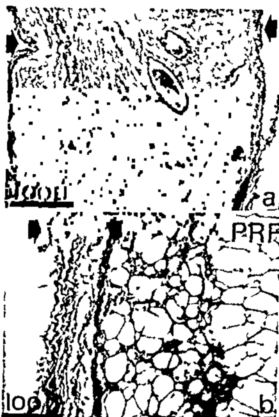


Fig 6 Case no 1 a Part of the capsule from the first transplanted kidney around which the perirenal fibrosis was identified The capsular tissue (between arrows) is very thickened fibrosed and contains several vessels Focally, a slight inflammatory reaction is present No foreign body reaction is seen (*cf* Fig 16) (H and E × 115)

b Part of the capsule from the second transplanted kidney without perirenal fibrosis The thickness of the capsule (between arrows) is in the normal range, and peripherally to the capsule the perirenal fat tissue is found (PRF) (H and E, × 160)

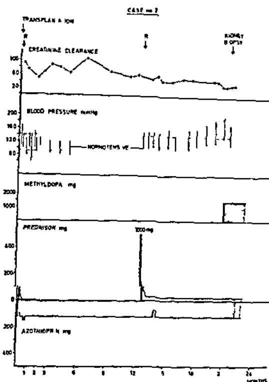


Fig 7 Diagram showing creatinine clearance, blood pressure and antihypertensive as well as immunosuppressive treatment in case no 2

The rejection crisis clinically suggested (R) and the time of biopsy are indicated by arrows The kidney function is found to be slowly decreasing, while there is a gradual rise in blood pressure, irrespective of the immunosuppressive treatment (*cf* Figs 1 and 11)

Kidney morphology A surgical biopsy of the renal capsule and the superficial kidney cortex was made 22 months after the transplantation The renal capsule was severely thickened and contained calcium deposits close to the renal parenchyma (Figs 9 and 10) In the superficial part of the renal cortex significant atrophy and interstitial fibrosis was present parallel to the findings in case no 1 (see Figs 3 and 4) Somewhat deeper in the renal cortex only a few nephrons without significant degenerative changes were present in the biopsy No morphological signs of immunological complications were found No epithelioid cell hyperplasia was found in the juxtaglomerular apparatus from the atrophic part of the parenchyma

Case no 3 30 year old man with terminal uremia and hypertension caused by chronic glomerulonephritis

Transplantation was performed with a necro-kidney with one major incompatibility Bilateral nephrectomy was not done

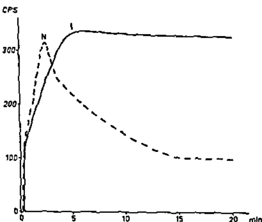


Fig 8 Case no 2 Curve I The renogram 12 months after transplantation shows a very high but delayed phase of uptake, an indistinct maximum peak 5 minutes after the injection followed by an extremely slow phase of elimination The findings suggest the presence of a diminished blood flow through the kidney but without decreased tubular uptake of hippuran

N is the renogram used as a reference (*cf* Fig 2) The reference in Figs 8 and 12 is different from that used in Fig 2 due to the use of a different apparatus

Due to a fever of long duration, rejection was suspected clinically and treated by increasing amounts of steroids 1 and 6 weeks after the transplantation (Fig 11) In spite of this creatinine clearance declined from 35 ml/min to 20 ml/min and during the next half year to 15 ml/min In the same period the hypertension of the patient increased from about 150/100 to 150/120

A second transplantation was done with a necro kidney with one possible, major HLA incompatibility Creatinine clearance now increased rapidly to 50 ml/min and the blood pressure was normalized (120/80) The first transplanted kidney was removed 3 months after the second transplantation At the operation severe thick fibrous adhesions were present between the transplant and the surrounding tissue

Isotype renography made at intervals of 2 6 days showed a nearly normal renogram 6 weeks after the transplantation (Fig 12 curve I)

Later the renograms showed a decrease in uptake a delayed peak and a progressively delayed excretory phase (Fig 12, curve II, *made 9½* months after the transplantation)

Kidney morphology At nephrectomy 9 months after the first transplantation the kidney was found to be somewhat contracted *with a total* renal volume of 65 ml The total *cortical volume* was 38 ml A severely thickened fibrous capsule around the kidney was noticed at operation As in case no 1 and 2 in the superficial part of the renal cortex significant tubular atrophy and interstitial fibrosis were present Some of the glomeruli were totally hyalinized (Fig 13) Deeper in the cortex round cell infiltration was frequently observed around the interlobular vessels, and in the arteries varying intimal thickening causing focal stenosis of the vessel could be found (Fig 14) Thus moderate signs of chronic rejection were found in the parenchyma Besides, moderate chronic hypertensive like changes of the arterioles were frequently seen A varying degree of epithelioid cell hyperplasia could be found in a part of the juxtaglomerular apparatuses

EXPERIMENTAL PERIRENAL FIBROSIS

In the 4 rats in which unilateral perirenal fibrosis was induced, the left kidney was found to be atrophic to a varying degree (Fig 15), and surrounded by a thick fibrous capsule in which foreign



Fig 9 Case no 2 The renal capsule (between big arrows) is severely fibrosed and thickened with a focal calcification close to the renal parenchyma (*cf* Figs 3 and 4) The *small* arrow in Fig 9 points radially out to the surface of the kidney, (H and E, × 40)



Fig 10 Case no 2 Higher magnification of Fig 9 In the cortex corticis severe fibrosis is seen between the tubules (arrows) (H and E × 80)

body reaction was observed (Fig 16) I 2 cases a perirenal abscess was seen at autopsy Superficially in the renal cortex some parenchymal atrophy was commonly found near the fibrotic capsule In the contralateral unaffected kidney no degenerative changes were present superficially in the cortical parenchyma (Fig 17)

DISCUSSION

Previously, unilateral perirenal fibrosis in patients has occasionally been seen as a complication to perinephritis or to perirenal hemorrhage (*e g Massumi 1960*) As an experimental parallel Page (1939) induced unilateral perirenal fibrosis in rats by enclosing the kidney in cellophane In case of unilateral affection this condition may sometimes give rise to hypertension of renal origin Uremia does not appear due to the function of the contralateral unaffected kidney

In patients with only one renal transplant the renal function obviously is solely dependent on this kidney Such patients also are much more sensitive to hypertension in case of pathologic changes of the transplant

In the three cases here described a significant perirenal fibrosis has been demonstrated either by biopsy (cases 1 and 2) or at the removal of the transplant (case 3) No renal artery stenosis vascular thrombosis, pyelonephritis, or transmitted glomerulonephritis were present Morphological evidence of moderate chronic rejection (Fig 14) was found only in one case (no 3)

In the clinical course of the 3 patients,

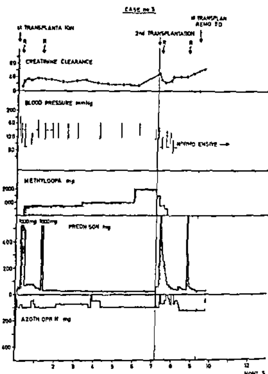


Fig 11 Diagram showing creatinine clearance blood pressure and antihypertensive as well as immunosuppressive treatment in case no 3

The rejection crisis clinically suggested (R) and the time of biopsy are indicated by arrows

After the first transplantation the kidney function is found to be gradually decreasing and the hypertension persists independent of the immunosuppressive treatment After the second transplantation a rapid decrease in blood pressure to normal values are found parallel to the increase in total kidney function (*cf* Figs 1 and 7)

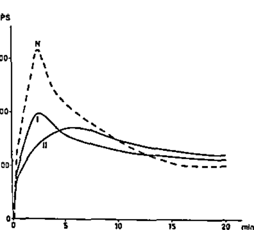


Fig 12 Case no 3 Curve I Renogram 2 weeks after transplantation A slightly decreased hippuran uptake is found with a maximum at 3 min after the hippuran injection, followed by a nearly normal elimination phase

Curve II Renogram 8 months after transplantation shows a very slow and diminished uptake to a delayed maximum peak 6 min after the injection Slow phase of elimination

The curves indicate a progressing reduction of the blood flow and a moderate decrease of the tubular uptake of hippuran, indicating some functional diminution of the tubules N reference value, see Fig 2

Fig 13 Case no 3 Biopsy from the transplanted kidney with perirenal fibrosis (the capsule is not shown in the figure) As in case 1, significant atrophy and fibrosis are present in the superficial part of the cortex (*cf* Figs 3, 9 and 10) (PAS-stain, × 70)

some common characteristics were found to exist (Figs 1, 7 and 11)

1) *Kidney function* The kidney function slowly declined within 1 year as estimated by the creatinine clearance Initially, the renal function was good in cases 1 and 2, but never rose to acceptable values in case 3 The decreased kidney function did not respond upon increase in immunosuppressive treatment in any of the patients, although in case 3 two

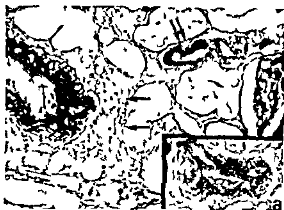


Fig 14 Case no 3 In the kidney cortex round cell infiltration is present (arrows) around an interlobular artery (I) In an afferent arteriole hypertensive changes of the vascular wall are found (double arrow) Many epithelioid cells are seen in the arteriole close to the glomerulus (PAS stain × 700)

Inset Interlobular artery from the same kidney in which the intima is greatly thickened causing stenosis of the vascular lumen (× 280)



Fig 15 The kidneys from a rat with unilateral perirenal fibrosis The affected kidney is considerably atrophied and in the contralateral kidney compensatory hypertrophy is found The stiff fibrotic capsule around the affected kidney is adherent to the renal cortex

clinically typical rejection episodes were present early in the clinical course At removal in the kidney histological signs of chronic vascular rejection were present to a moderate degree

2) *Hypertension* In all cases the hypertension preceded the severe decrease in kidney function According to *Page* (1939) experimental perirenal fibrosis of one kidney gives rise to a more frequent and severe hypertension in case of contralateral nephrectomy than if the unaffected kidney remains in the animal It is interesting to note that the antihypertensive effect of a well functioning kidney is well demonstrated in case 3 (Fig 11) This is in accordance with the experimental evidence of the lowering effect a normal kidney has on blood pressure in nephrogenic hypertension (*Fasciolo,* 1938, *Gomez et al* 1959)



Fig 16 Rat kidney with perirenal fibrosis Beneath the thick fibrotic capsule the renal parenchyma is found to be atrophic as compared to the contralateral kidney from the same animal (*cf* Fig 17) Near the fibrotic capsule the cortical atrophy is most pronounced (arrows) (V G eson stain × 45)

Inset In the fibrotic capsular tissue the inflammation and foreign body reaction found is caused by the talc powder (Phase contrast × 680)



Fig 17 The *unaffected* kidney from rat with unilateral perirenal fibrosis In the kidney no atrophy or fibrosis is found (V Gieson stain, × 45)

3) *Morphological parallels in clinical and experimental perirenal fibrosis* By comparing the biopsies from the 3 patients with the kidneys from rats with experimental perirenal fibrosis it is seen that an essentially identical morphological picture was found The atrophic changes of the kidney cortex were most pronounced in the superficial part in both patients and animals, and an equal decrease in the weidth of the cortex corticis was found As a rule, the glomeruli were vascularized in spite of the great tubular atrophic changes in this part of the cortex. Deeper in the cortex the parenchyma was affected only to a very slight degree by the capsular thickening

4) *Functional implications of perirenal fibrosis in transplanted kidneys* It remains to be definitely established to which degree the fibrosis of the renal capsule causes the diminishing in kidney function in the present investigation It seems fair to conclude, that in cases 1 and 2, the fibrosis has been the main cause of the functional decrease In case 3 the conditions are less clear due to the initial rather low function and to the signs of rejection found both clinically and morphologically. Here, the perirenal fibrosis is just one out of several pathological processes being present in the transplanted kidney

As the appearance of perirenal complications (abscess, bleeding, etc) is more frequent in transplanted kidneys than otherwise, the development of a significant perirenal fibrosis is probably not an uncommon complication in human renal transplantation As the renal function in this group of patients is solely maintained by the transplanted kidney, such patients are much more sensitive to the development of nephrogenic hypertension caused by the development of perirenal fibrosis In accordance, *Braasch et al* (1942), in a group of non transplanted patients having unilateral perirenal fibrosis with no affection of the contralateral kidney, found only 4 per cent of nephrogenic hypertension in this series

---

This work was supported by *Statens almindelige Videnskabsfond, Foreningen til Hjertesygdommenes Bekæmpelse* and *P A Brandts Fond*

REFERENCES

Brasch, W F & Wood, W W Clinical Peri nephritis and Blood Pressure Proc Staff Meet Mayo Clin *17* 52–54, 1942

Faarup, P & Petri, M Peroperative Evaluation of the Juxtaglomerular Apparatus in Human Kidneys Acta path microbiol scand *76* 326–327, 1969

Fasciolo, J C Accion del rinon sano sobre la hipertension arterial por isquemia renal Rev Soc argent biol *14* 15–27, 1938

Gomez, A H, Hoobler, S W & Blaquier, P Effect of Addition and Removal of a Kidney Transplant in Renal and Adrenocortical Hypertensive Rats Circ Res *8* 464–472, 1960

Hegedus, V & Faarup, P The Cortical Volume of the Normal Human Kidney Correlated Angiographical and Morphological Studies Acta Radiol Scand (in press)

Massumi, R A, Andrade, A & Kramer, N Arterial Hypertension in Traumatic subcapsular Perirenal Hematoma (Page Kidney) Evidence for Renal Ischemia Amer J Med *46* 635–639, 1969

Page, I H The Production of Persistent Arterial Hypertension by Cellophane Perinephritis J A M A *113* 2046–2048, 1939

Acta path microbiol scand Section A 81, 47–56 1973

ALLOXAN DIABETES IN DUCT-LIGATED RATS

Light and Electron Microscopic Findings

CURT EDSTRÖM and LENNART BOQUIST

Institute of Pathology (Head Prof S Falkmer), University of Umeå, S 901 87 Umeå Sweden

The course of alloxan diabetes was studied ultrastructurally during 19 months in duct ligated and control rats During the first days after alloxan administration, selective degeneration was observed in most β cells of both atrophic and intact pancreatic parenchyma New formation of islet cells was found from about one week Two different kinds of cells without characteristic granulation were seen those which seemed to be precursor cells to granulated islet cells and those which appeared to represent hypertrophic, degranulated β cells The number of α_2 cells was increased during the first months after the injection of alloxan At 19 months a normal fine structure was found in the islets

In preceding parts of our studies on duct ligated rats (*Edström* & *Falkmer* 1967, 1968, *Boquist* & *Edström* 1970), the alloxan sensitivity (*Edström* 1971 a, *Edstrom* & *Hassler* 1971) and long term morphological and functional changes evoked by diabetogenic doses of alloxan were reported (*Edström* 1973) The effects of alloxan in duct ligated rats differed from those in non diabetic Chinese hamsters (*Boquist* 1968 a) in that the glucose tolerance curves still were of diabetic type 8 months after the administration of alloxan in rats whereas a complete restitution was obtained in Chinese hamsters already within 4 weeks after the injection In the Chinese hamster islets a series of structural alterations could be correlated with the various phases of the functional restitution, including the appearance of sparsely granulated cells ('agranular" cells) (*cf Boquist* & *Falkmer* 1970) which gradually seemed to be transformed into mature β cells Similar sparsely granulated cells were also observed in duct ligated rats, both in the early (*Edström* 1971 a) and late (*Edström* 1973) phase after alloxan injection These cells showed some paradoxical argyrophil staining reactions and it could not be ascertained whether they were young, immature cells, or merely degranulated β cells Therefore, the light microscopic studies have been supplemented by the present ultrastructural examination which also allows a more detailed comparison with the previously reported regenerative phenomena in the Chinese hamster (*Boquist* 1968 a b)

Received 3 viii 72 Accepted 14 viii 72

Requests for reprints should be addressed to Dr *Curt Edström* Institute of Pathology, University of Umeå *S 901 87 Umeå* Sweden

MATERIAL AND METHODS

Data about the animals and the experimental procedures are given in the preceding reports (*Edström* 1971 a b 1973) In all light microscopic examination was performed on the pancreas from 18 duct ligated and 3 control rats, and the pan

creas from 15 of these duct ligated and all the 3 control animals was also examined ultrastructurally The animals were killed at the following time intervals after intraperitoneal alloxan administration 15 and 60 minutes, 1 and 2 days, 1, 2, 4 and 5 weeks, 2, 3, 5, 8 and 19 months One or two animals were investigated at every observation time The rats were the same as in the preceding study (*Edström* 1973)

Specimens were taken from the atrophic and intact pancreas of subtotally duct ligated rats and from the head of the pancreas of alloxan diabetic, sham operated animals for the following morphological procedures

Light microscopy Specimens fixed in Bouin's fluid were stained with van Gieson's stain, chrome haematoxylin ponceau fuchsin aldehyde fuchsin and the silver impregnations according to *Hellerström* & *Hellman* (1960) and *Grimelius* (1969)

Electron microscopy The specimens were fixed by immersion in 1 per cent osmium tetroxide in 0 34 M Veronal acetate buffer adjusted to pH 7 4 and were then embedded in Epon 812 In addition, a few pancreatic specimens were processed according to *Grimelius* (1969) for the ultrastructural identification of silver particles in the islet parenchymal cells The technical quality of the sections obtained in this way was acceptable, but not very high The reason for this was probably that we used Bouin fixation and Epon embedding instead of the formalin fixation and Vestopal-embedding recommended by *Grimelius (personal communication)* Thick sections stained with toluidine blue were used for identification of appropriate areas for the thin sections The sections were stained with uranyl acetate and lead citrate and were then examined in Siemens Elmiscopes IA and 101

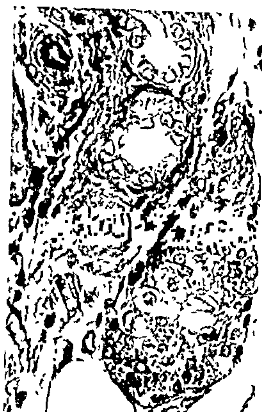


Fig 1 Photomicrograph of atrophic pancreatic portion in duct ligated rat showing ductules and a so-called islet body with a few large cells without cytoplasmic granulation Aldehyde fuchsin stain × 80

RESULTS

As mentioned in the Introduction survey descriptions of the morphological alterations in the pancreas of the alloxan-diabetic, duct ligated rats have already been given (*Edström* 1971 a, 1973) Therefore, the present report is focussed on the light and electron microscopic observations made in duct-ligated rats from one week to 19 months after the administration of alloxan, as these are the time intervals during which the "agranular" cells seem to arise and gradually become transformed into mature β cells

15 minutes—2 days During the first two days selective degeneration of the β cells was observed, leaving only some necrotic *debris* No obvious differences in alloxan induced alterations between atrophic and intact pancreatic parenchyma were observed

1 week Light Microscopy Several so-called islet bodies (*Edström* & *Falkmer* 1967) were found in the atrophic pancreatic portion close to or in direct contact with the ductules (Fig 1) The islet bodies were similar to those previously observed (*Edström* 1973) and contained a varying number of cells without characteristic granulation ('agranular' cells)

In the islets of both intact and atrophic parenchyma and in the islets of the control rats there was necrotic *debris* in the central portion of the islets It was difficult by light microscopy to ascertain whether any un

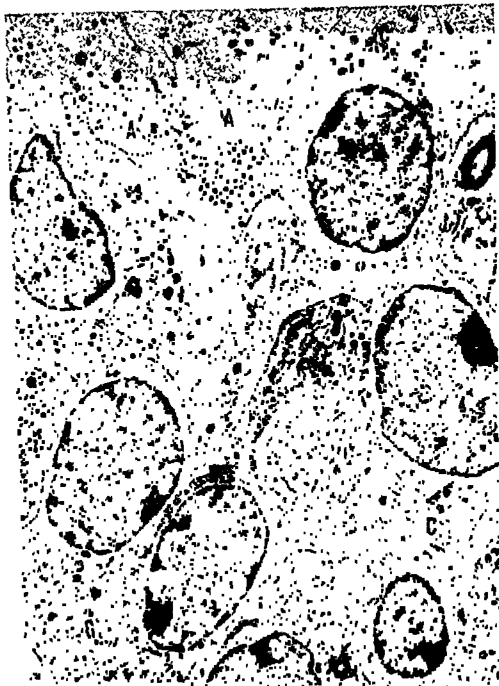


Fig 2 Electron micrograph of pancreatic islet showing α_2 cells (A) with dense secretory granules and β-cells with well developed Golgi complex (G) and an occasional giant mitochondrion (M) There are also cells with low cytoplasmic electron density, moderately developed cytoplasmic organelles and only a few granules of β type (C) $\times$ 4 000



Fig 3 Electron micrograph showing a large cell with peripheral nucleus and moderately developed cytoplasmic organelles *Vesicles vacuoles and lysosome like bodies are seen but there are no typical secretory* granules Some of the surrounding cells possess a few secretory granules of a_2 type × 10 000

damaged β cells persisted Large cells without characteristic granulation were found in the central portion of some islets both in those with and in those without necrotic *debris* The α and α cells appeared unaffected

Electron Microscopy The ultrastructural examination was mainly concerned with the cells which light microscopically were devoid of characteristic granulation It seemed that there were two kinds of such cells They were tentatively called agranular cells type I and agranular cells type II Intermediate forms between these cell types were also found

The agranular cells type I (Fig 2) were of small or moderate size had a rounded or oval nucleus with uneven chromatin distribution and a moderately large nucleolus The cytoplasm possessed low electron density scattered free ribosomes sparsely developed endoplasmic reticulum of vesicular or lamellar type and a few rounded or oval and rather small mitochondria The size of the Golgi complex varied Some of these cells were devoid of secretory granules whereas others contained a few granules often close to the Golgi complex Occasional granule cores occurred in the Golgi cisterns The structural appearance of the secretory granules varied most of them were of β type but some were of α type and a few of α_1 type

The agranular cells type I were mainly found close to the ductules They occurred in the buds on the ductules and in the islet bodies often in an intermediate position between the ductule epithelial cells and the peripheral granulated endocrine cells Rather few of these cells were seen in the islets

The agranular cells type II (Fig 3) were usually large and had a rounded nucleus that often was peripherally localized The nuclear chromatin was somewhat unevenly distributed and the nucleolus was large In the cytoplasm there were numerous free ribosomes in a ground substance of low density The endoplasmic reticulum was prominent rough surfaced and of lamellar or more seldom of vesicular type The Golgi complex was well developed and contained occasional granule cores A moderate number of elongated mitochondria of moderate size were found There were rather few secretory granules with a core of low or moderate density and a rather wide space between the membrane and the granule core They were interpreted as β granules and occurred both in the Golgi region and in other portions of the cytoplasm often close to the cell membranes Signs of emiocytosis were seen in a few of these cells

The agranular cells type II were not seen in direct contact with the ductules or in the small buds on the ductules but a few cells of this type occurred in the islet bodies Most often these cells were encountered in the central portion of the islet

In the islets there were also a few remaining β cells without degenerative changes They possessed a prominent Golgi complex and endoplasmic reticulum a great number of mitochondria and a varying number of secretory granules (Fig 4)

2 weeks Light Microscopy The atrophic parenchyma exhibited proliferating ductules with associated buds and islet bodies that contained a varying number of cells without characteristic granulation The islets of both atrophic and intact parenchyma and of control rats exhibited an increased number of α cells mainly of α type Islets were found which were almost entirely composed of α cells a few α_1 cells and a varying number of centrally situated large cells without characteristic granulation

Electron Microscopy The light microscopic observation of the presence in the islets of a great number of α cells was confirmed The α cells possessed secretory granules with a core of low density and a closely applied membrane (Fig 5) More or less characteristic agranular cells type II were found in the islets and agranular cells type I occurred in the islet bodies A few typical β cells with well developed cytoplasmic organelles were present in the islets

1–5 months Light Microscopy As com

4*

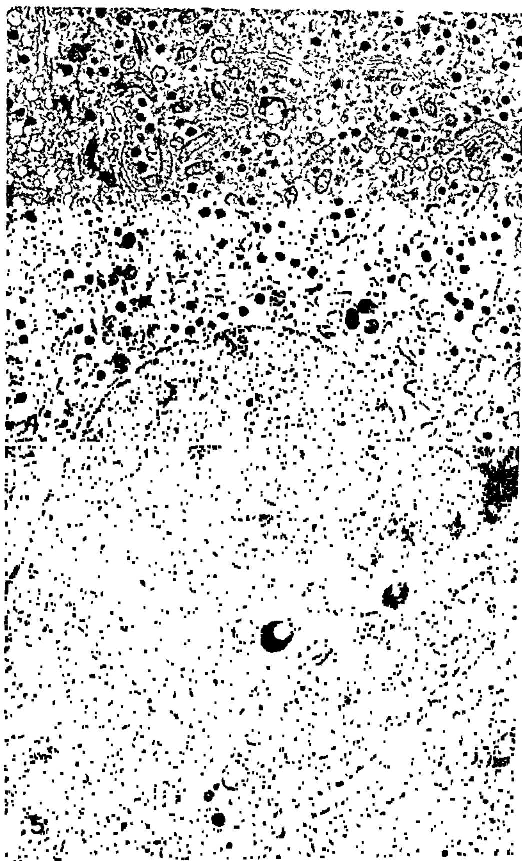




Fig 6 Photomicrograph of consecutive sections of pancreatic islet showing that the large central cells are not stained by aldehyde fuchsin (A) but are slightly stained by Grimelius' silver stain (B) × 100

pared with the findings at 2 weeks, an increased number of cells without characteristic granulation was found in the central portion of the islets When consecutive sections were stained with aldehyde-fuchsin, chrome alum-haematoxylin, and Grimelius' silver stain, respectively, it was found that the large cells were not stained with aldehyde fuchsin (Fig 6 A) and chrome alum haematoxylin, but were slightly stained with a brown or green tinge with Grimelius' silver stain (Fig 6 B)

Electron Microscopy Agranular cells type II were found in the central portion of the islets and occasional agranular cells type I were seen which contained only a few secretory granules of α_2-type

8 months Similar findings were made as at 1–5 months However, the number of normal appearing β-cells was greater Grimelius' silver stain showed still brown or green coloration of the central cells in some islets

19 months Both in the light and electron microscopes it was found that the pancreatic islets of atrophic and intact parenchyma and of control rats seemed to be normal As compared with the observations at 8 months, the number of β-cells was increased and the number of α-cells and agranular cells type I and II was decreased The β cells showed normal ultrastructural features In a few β cells there were intranuclear rods and so-called nuclear bodies of similar type as previously described (*Boquist* 1969, 1970) The fine structure of the α_1- and α_2-cells was normal

The ultrastructural examination of sections processed according to *Grimelius* (1969) showed silver particles localized to secretory granules in α_2- and a few α_1-cells Silver particles were not clearly observed in mature β-cells, but were occasionally seen in the cytoplasm of some agranular cells type I and II It was not possible to ascertain whether the distribution and concentration of the

Fig 4 Islet β-cells showing typical secretory granules, a well developed Golgi complex and elongated mitochondria × 11 000

Fig 5 α_1-cell in pancreatic islet possessing numerous secretory granules with a core of low density and a closely applied membrane × 16 000

silver particles varied in different kinds of granules and whether they were homogeneously or irregularly distributed in the granule cores and/or membranes

DISCUSSION

The morphological findings in the preceding (*Edström* 1971 a, 1973) and present works show that alloxan induces a selective destruction of the β cells in both the atrophic and intact portions of the pancreas of duct ligated rats. Any previous morphological documentation of the effects of alloxan on duct ligated animals has not been presented. *Zweens & Bouman* (1967) who studied the effects of duct ligation after alloxan administration to rats reported functional changes and islet new formation but did not give any detailed description or any illustration of the morphological alterations. In the preceding work (*Edström* 1973) it was found that the early effects on the fasting blood glucose level of alloxan in rats duct ligated 5–6 weeks after injection of alloxan were more marked than those observed in sham operated animals. Any corresponding difference in the morphology of the pancreas in the two groups of animals was not found in the present study.

Although there was a selective destruction of the β cells after alloxan administration there was no complete disappearance of all the β cells. The few β cells which persisted showed signs of high functional activity which we interpret as an effort of the organism to compensate for the great loss of insulin producing parenchyma.

The great number of α cells observed from the second week after alloxan injection may represent a relative increase due to the decreased number of β cells. However, the possibility of a real new formation of α cells should also be considered. Islets composed almost entirely of α cells have been observed in alloxan treated mice (*Patent & Alfert* 1967) and in streptozotocin treated guinea pigs (*Petersson et al* 1970) and rats (*Steiner et al* 1970). Also in the present study there were islets with a predominance of α cells. Inasmuch as there also were secretory granules of α and α type in the agranular cells type I of the islet bodies and granules of α type in some cells of similar type in the islets we believe that there was a real new formation of α cells mainly of α type in addition to a relative increase of these cells in the islets. The cause for the supposed new formation of α cells in the alloxan diabetic animals is not known. However, there may be a stimulation of the α cells on the few remaining β cells as has been suggested for streptozotocin treated animals (*Steiner et al* 1970).

The agranular cell types varied as to structural features and localization. The agranular cells type I were seen early after the injection of alloxan and were mainly associated with the ductules often with a tendency to localization between central ductule epithelial cells and peripheral granulated endocrine cells. In the latter respect they were similar to the sparsely granulated cells which have been observed in the endocrine pancreas of newborn rodents (*Boquist* 1972 a) and after plain duct ligation in rats (*Boquist & Edström* 1970) as well as in the regenerative phase of alloxan diabetes in the Chinese hamster (*Boquist* 1968 a, b). They have features in common with the agranular cells observed in states of new formation of islet cells (*cf. Boquist & Falkmer* 1970) and may thus represent precursor cells. The presence not only of β granules but also of α and a few α granules in these cells indicates that they might differentiate into various islet parenchymal cell types. This gives interesting aspects on the biological nature of these cells.

The agranular cells type II do not seem to be precursor cells. We believe that they represent hypertrophic degranulated β cells without any ability or opportunity to store insulin in secretion granules. Similar seemingly hypertrophic and degranulated cells have been observed in Mongolian gerbils with islet hyperplasia and obesity (*Boquist* 1972 b).

The results of the silver impregnations of light and electron microscopic sections according to *Grimelius* (1969) do not allow any further interpretations. Usually, these impregnations give a positive reaction in α_1- and α_2-cells with considerable species variations. However, a positive staining with this technique may also be obtained in some β-cells (*van Assche* & *Gepts* 1971). As far as can be concluded from the present results, it seems that the brown or green tinge in silver impregnated light microscopic sections may be a feature both of agranular cells type I and II.

The finding at 19 months of normal appearing islets denotes that there has been a morphological restitution of the endocrine pancreas in the alloxan-treated animals. It is known from the work of *Lazarow* (1952) that a functional amelioration after alloxan administration occurs in non-ligated rats after about 1–2 years. In the preceding work (*Edström* 1973) it was not settled whether a functional normalization had occurred at 19 months also in alloxan-treated, duct-ligated rats, although the fasting blood glucose level was normal. When regeneration of islet parenchyma occurs after alloxan injection in laboratory animals, it usually starts already 1 or 2 weeks after the injection (*Boquist* 1968 a, b). The findings of early proliferation of ductules and early appearance of agranular cells (see above) in the presently studied animals, denote that there is an early new formation of islet parenchyma also in these duct-ligated rats. This newly formed parenchyma seems to have a good (*Zuccens* & *Bouman* 1967) though not sufficiently high functional capacity, as evidenced by the return of the blood glucose level to the normal range and by the persistence of decreased glucose tolerance after 5 months (*Edström* 1973). The finding of a few persisting highly active β-cells (see above) lends morphological support to the supposition of a high activity in the endocrine pancreas of the alloxan-treated, duct-ligated rats.

Supported by grants from the *Swedish Medical Research Council* (Project No B72-12X-718-07A), the *Medical Faculty of Umeå* and the *Swedish Diabetes Association*.

REFERENCES

van Assche, F. A. & *Gepts, W.*: The cytological composition of the foetal endocrine pancreas in normal and pathological conditions. Diabetologia *7*: 434–444, 1971.

Boquist, L.: Alloxan administration in the Chinese hamster. I. Blood glucose variations, glucose tolerance, and light microscopical changes in pancreatic islets and other tissues. Virchows Arch. Abt. B Zellpath. *1*: 157–168, 1968 a.

Boquist, L.: Alloxan administration in the Chinese hamster. II. Ultrastructural study of degeneration and subsequent regeneration of the pancreatic islet tissue. Virchows Arch. Abt. B Zellpath. *1*: 169–181, 1968 b.

Boquist, L.: Intranuclear rods in pancreatic islet β-cells. J. Cell Biol. *43*: 377–381, 1969.

Boquist, L.: Some peculiar fine structures in the pancreatic islets of diabetic and non-diabetic rodents. Acta Diabetol. Lat. *7*: 590–615, 1970.

Boquist, L.: The fine structure of the endocrine pancreas in newborn rodents. Diabetes. In press, 1972 a.

Boquist, L.: Obesity and pancreatic islet hyperplasia in the Mongolian gerbil. Diabetologia *8*: 274–282, 1972 b.

Boquist, L. & *Edström, C.*: Ultrastructure of pancreatic acinar and islet parenchyma in rats at various intervals after duct ligation. Virchows Arch. Abt. A Path. Anat. *349*: 69–79, 1970.

Boquist, L. & *Falkmer, S.*: The significance of agranular and ciliated islet cells. In: Falkmer, S., Hellman, B. & Täljedal, I. B. (eds.) The Structure and Metabolism of the Pancreatic Islets. A Centennial of Paul Langerhans' Discovery. Pergamon Press, Oxford 1970, pp. 25–35.

Edström, C.: Alloxan sensitivity of rats at various intervals following ligation of the pancreatic ducts. Acta Soc. Med. Upsal. *76*: 77–86, 1971 a.

Edström, C.: Further quantitative structural studies of the pancreatic islet parenchyma in rats with duct ligation. Acta Soc. Med. Upsal. *76*: 127–138, 1971 b.

Edström, C.: Course of alloxan diabetes in duct-ligated rats. A functional and morphological study. Acta path. microbiol. scand. *81A*: 21–35, 1973.

Edström, C. & *Falkmer, S.*: Qualitative and quantitative morphology of rat pancreatic islet tissue five weeks after ligation of the pancreatic ducts. Acta Soc. Med. Upsal. *72*: 376–390, 1967.

Edstrom, C & *Falkmer, S* Pancreatic morphology and blood glucose level in rats at various intervals after duct ligation Virchows Arch Abt A Path Anat *345* 130–153, 1968

Edström, C & *Hassler, O* Functional capacity, alloxan sensitivity and vascular supply of pancreatic islet parenchyma during the first week after duct ligation Acta Soc Med Upsal *76* 211–222, 1971

Grimelius, L Studies of adult human pancreatic islet cells with a new silver nitrate stain Abstr Upsal Diss Med *62* 1–23, 1969

Hellerström, C & *Hellman, B* Some aspects of silver impregnation of the islets of Langerhans in the rat Acta Endocr *35* 518–532, 1960

Lazarou, A Spontaneous recovery from alloxan diabetes in the rat Diabetes *1* 363–371, 1952

Patent, G J & *Alfert, M A* Histological changes in the pancreatic islets of alloxan treated mice, with comments on the β cell regeneration Acta Anat (Basel) *66* 481–493 1967

Petersson, B, *Hellerstrom, C* & *Gunnarsson, R* Structure and metabolism of the pancreatic islets in streptozotocin treated guinea pigs Horm Metab Res *2* 313–317, 1970

Steiner, H, *Oel, O*, *Zahnd, G* & *Froesch, E R* Studies on islet regeneration hyperplasia and intrainsular cellular interrelations in long lasting streptozotocin diabetes in rats Diabetologia *6* 558–564 1970

Zueens, J & *Bouman, P R* Neoformation of insulin producing islets following ligation of the pancreatic ducts in normal and alloxan diabetic rats Acta Physiol Pharmacol Neerl *14* 529 530 1967

Acta path microbiol scand Section A 81, 57 63, 1973

RETICULUM CELL NEOPLASMS IN NORMAL AND BENZENE TREATED HAIRLESS MICE

OLF DIDRIK LÆRUM

Institute of Pathology, Section B, University of Oslo, Rikshospitalet, Oslo 1
Head Olav Hilmar Iversen

The occurrence of reticulum cell neoplasms in normal and benzene treated hairless mice (hr/hr) was investigated 39 per cent of a group of untreated mice had these neoplasms at a mean age of about 20 months The same tumour yield was also found in another group of mice which were painted twice weekly on the back skin with benzene up to a mean age of 20 months The histological patterns of reticulum cell neoplasms characteristic of these mice are described

Benzene is known as a leukaemogenic agent to animals as well as man (*Browning* 1965), but is not carcinogenic to the skin Recently we have used benzene as solvent in a study of the effects of some weakly carcinogenic agents on the hairless mouse skin (*Lærum* & *Iversen* 1972) We found that the vesicant agent cantharidin was weakly carcinogenic to the epidermis and in addition increased the frequency of reticulum cell neoplasms to nearly 60 per cent In a group of mice which were treated with repeated benzene applications after previous initiation with the carcinogen 20 methylcholanthrene, about 30 per cent of the animals developed these lesions

Therefore, we felt the need for a more thorough investigation of the effects of repeated applications of benzene to the skin of hairless mice It was of special interest to study the frequency of reticulum cell neoplasms in benzene treated mice where no previous carcinogenic initiation had been performed

Received 4 x 72 Accepted 8 x 72

It is known that reticulum cell neoplasms may have a very high spontaneous frequency, rising with increasing age (*Dunn* 1954), in different strains of mice including a strain of hairless mice (*Deringer* 1951) It was therefore of importance also to investigate the spontaneous frequency and histological types of reticulum cell neoplasms in our strain of hairless mice, especially as this strain has been widely used for studies of carcinogenesis (*Iversen* & *Evensen* 1962, *Elgjo* 1968, *Lærum* 1972)

MATERIALS AND METHODS

Hairless mice of the strain hr/hr, equal numbers of each sex, were used These mice have been bred in our laboratory since 1939 and are described by several workers (*Crew* & *Mirskaia* 1932, *David* 1932, *Iversen* & *Evensen* 1962, *Iversen* & *Iversen* 1967, *Lærum* & *Bjerknes* 1972)

At the beginning of the study, groups of hairless mice were taken randomly from a larger pool of animals, 6 to 8 weeks of age, and kept in plastic cages, 5 mice in each The animals were given a continuous water supply and a standard diet (mouse food pellets, Felleskjøpet a/s, Oslo) The total number was 71 mice

One half of the animals were painted regularly twice weekly on the back skin with benzene (Benzolum liq pro analysi, Riedel de Haën, Hannover) using a small brush (Group I)

The other half of the mice were not given any treatment (Group II), but were observed for up to 2 years Autopsies were performed on all animals Some mice which succumbed to intercurrent, non tumourous diseases before the first tumour appeared, e g 5 months, were not included in the material

The *reticulum cell neoplasms* were typed according to the classification of *Chouroulinkov et al* (1964)

RESULTS

The results are shown in Tables 1 and 2 It is seen that benzene caused a few benign skin papillomas in 3 animals (8 6 per cent) Otherwise the tumour frequency was about the same in the benzene painted and the untreated animals 38 per cent of the total number of animals had various kinds of reticulum cell neoplasm including reticuloses malignant lymphomas and leukaemia The average age of animals with these tumours was 18 5 months (not shown in the Table) which is only slightly less than the average for all the mice (Table 1) Other malignant tumours were found in 8 5 per cent of the animals These included among others one pulmonary carcinoma and one endometrial sarcoma

TABLE 1 *Tumours in Hairless Mice after Benzene Application (Group I) and in Untreated Animals (Group II)*

	Group I	Group II	Total
Number of mice	35	36	71
Age in months ± SD	19 8±5 6	19 6±4 8	19 7±5 1
Per cent with tumours			
Reticulum cell neoplasms (total)	37 1	38 9	38 0
Reticuloses	22 9	33 3	28 2
Malignant lymphomas	8 6	5 6	7 1
Leukaemia	5 7	0	2 8
Others			
Malignant	8 6	8 3	8 5
Benign	8 6	8 6	8 6
Skin papillomas	8 6	0	

TABLE 2 *Relative Distribution of the Histological Types of Reticuloses in Hairless Mice*

	A	B
Total number	20	48
Per cent		
Lymphoreticulosis	25	56
Reticulohistiocytosis	20	15
Reticuloendotheliosis	15	11
Reticulogranulomatosis	10	10
Reticulolymphogranulomatosis ('Type B')	30	8

A is the present material with normal and benzene treated mice B is taken from a study by *Lærum & Iversen* (1972), showing the frequency of reticuloses in the same strain of mice after skin application of various weak carcinogenic agents.

The most dominating lesions among the reticulum cell neoplasm were different kinds of reticuloses The relative distribution of these is shown in Table 2 A comparison with our earlier material published two years before is also made In the latter material, lymphoreticulosis was the most frequent type accounting for 56 per cent, while in the present study, reticulolymphogranulomatosis ('Hodgkin-like lesion') was the most frequent, accounting for 30 per cent The histological types in our present material, together with a brief description are shown in Figs 1–5

Fig 1 Lymphoreticulosis in the spleen The spleen was considerably larger than normal The picture shows a proliferation of lymphoid elements with a more or less atypical reticulum The regular follicular structure is lost and granulocytes and extramedullary haemopoiesis are more or less absent Haematoxylin erythrosin stain magnification × 289

Fig 2 Reticulohistiocytosis from a lymph node The regular structure is destroyed by infiltrating histiocytic cells The lesion is common in liver and

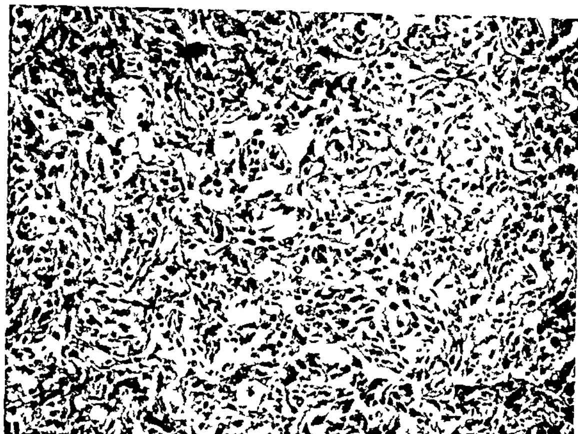


Fig 3 Reticuloendotheliosis in the spleen This is a proliferation of endothelial cells with formation of small lumina Sometimes also larger lumina with papillary growth are seen Liver and spleen are the most common localizations Haematoxylin erythrosin stain magnification × 289

If these lesions were of infectious origin it should be expected that mice in the same cage infected each other However if the neoplasms were grouped according to the cages of the respective animals an even distribution was found One to 2 cases of reticulum cell neoplasms were found in each cage of 5 mice except in one cage (Group II) where all 5 mice had different types of these lesions

DISCUSSION

As shown in Table I benzene had no obvious effect on the occurrence of reticulum cell neoplasms in the hairless mice after repeated applications to the back skin Two cases of myelogenous leukaemia were observed but this is not sufficient to indicate any specific effect These results are in contrast to those previously reported by *Lignac* (1932) who found that repeated injections of benzene was leukaemogenic to mice It is possible that the rate of resorption through the skin may be a limiting factor although benzene has a

Fig 4 Reticulogranulomatosis in the liver This is a more or less localized proliferation of reticular cells with infiltration of leucocytes which are often atypical Haematoxylin-erythrosin stain magnification × 289

Fig 5 Reticulolymphogranulomatosis in the liver These tumours have a strongly polymorphous pattern with proliferation of granulocytes lymphocytes reticulum cells and giant cells which may resemble Sternberg cells The neoplasm is similar to Hodgkin s disease in man except that eosinophilia and fibrosis are absent In Dunn s classification it is called Reticulum cell neoplasm type B *Haematoxylin-erythrosin stain magnification* × 289

high solubility for lipids and should thus easily penetrate the epidermis (*Scheuplein* 1971)

The frequency of reticulum cell neoplasms in hairless mice was about 30 per cent in benzene treated mice, also if the skin had been initiated with a small dose of 20 methyl cholanthrene (*Lærum & Iversen* 1972) Therefore, the spontaneous frequency of reticulum cell neoplasms in our mice does not seem to be affected by the very high amounts of benzene which were applied during their lifetime even after initial carcinogen treatment

Reticulum cell neoplasms had a high spontaneous frequency in our hairless mice among which about 39 per cent of the animals had these lesions at an age of nearly 20 months (Table 1) Other strains of mice may even have a higher frequency, such as a substrain of C57 BL among which 75 per cent of the animals had such neoplasms at the age of 20 months (*Dunn & Deringer* 1968) However, the number is mainly found to be lower (*Chouroulinkov et al* 1964) In *Deringer's* studies (*Deringer* 1951), 23 25 per cent of untreated hairless mice had reticulum cell neoplasms at the age of 24 months a value similar to that found in the present study (Table 1) As in our material (Table 2), reticulolymphogranulomatosis ("Type B) was the most frequent lesion On the other hand in our previous study (*Lærum & Iversen* 1972) where the mice had received various forms of carcinogenic treatment, lymphoreticulosis was the most common histological type (listed in Table 2) This indicates that the distribution of histological types may change if the frequency of these neoplasms is increased

The reticulum cell neoplasms described were typical lesions in old hairless mice These animals had an average age of 18 5 months and the first tumours appeared in mice of 5 6 months of age Most of the tumours were characteristic reticuloses and only a few were localized reticulosarcomas or lymphosarcomas (Table 2) For histological typing of the reticuloses the nomenclature of *Chouroulinkov et al* (1964) was found to be most useful This classification is based on the assumption that the reticuloses are only different aspects of diffuse tumour development in the reticuloendothelial system (see Figs 1–5)

The histological types of reticuloses characteristic of our mice are demonstrated in Figures 1–5 In most cases, several internal organs were affected, such as liver, spleen lymph nodes kidney and lungs The histological type varied from organ to organ and exhibited different patterns even within the same organ of one mouse The typing was therefore made according to the most dominating lesion

Several etiological agents have been proposed to be the cause of reticulum cell neoplasms in mice Strong carcinogens, such as 20 methylcholanthrene and 3,4 benzpyrene, as well as benzene, mineral oil and also Moloney virus have been reported to induce these lesions (*Deringer* 1951, *Dunn* 1954, *Dunn & Deringer* 1968, *Lignac* 1932)

The relative frequency of these neoplasms increases also after thymectomy (*Metcalf et al* 1966) In this connection it is interesting that *Burstein & Allison* (1970) found a shorter latency period for the development of reticulum cell neoplasms when SJL/J mice were treated with antilymphocytic serum

In our previous study (*Lærum & Iversen* 1972) we found that the frequency of these lesions was doubled when hairless mice after MCA initiation were painted regularly with cantharidin during their whole life span However, such a 'carcinogenic action is not specific when very high doses of the agent only cause a relative increase in the frequency of tumours which also occur spontaneously

A viral etiology of these lesions was strongly supported in a recent work by *Peters et al* (1972 a, b) They found high rates of C type RNA tumour virus gs antigen in the spleens of BALB/cCr mice bearing reticulum cell neoplasms The incidence of these neoplasms rose progressively with increasing age of the animals and at the same time the prevalence of the tumourvirus gs antigen

showed a parallel increase They could also isolate infectious virions of C type in more than one half of the animals over 6 months of age

A possible mechanism of the action of cantharidin might then be to lower the resistance of the animals to a latent viral infection The fact that the lesions were not restricted to certain animal cages, does not necessarily rule out an infectious origin of these neoplasms in our hairless mice

The following conclusions can be drawn

1 Our strain of hairless mice has a high spontaneous frequency of reticulum cell neoplasms with increasing age These are mostly generalized reticuloses which affect several organs

2 The spontaneous frequency of these neoplasms is not affected by applications of benzene when painted on the skin twice weekly during the whole life span of the animals

3 The most frequent type of reticulosis in our strain of hairless mice is reticulolymphogranulomatosis, resembling "Hodgkin's disease" in man

---

The author thanks Mrs *Kari Hansen* and Mrs *Aasa Schjolberg* for skilful technical assistance

REFERENCES

Browning, E Toxicity and Metabolism of Industrial Solvents Elsevier Publ Comp 1965

Burstein, N A & Allison, A C Effect of antilymphocytic serum on the appearance of reticular neoplasms in SJL/J mice Nature (Lond) 225 1139 1140, 1970

Chouroulinkov, I , Rivière, M R & Guérin, M Étude anatomopathologique des réticuloses de la souris J Reticuloend Soc 1 277–292, 1964

Crew F A E & Mirskaia, L The character 'hairlessness' in the mouse J Genet 25 17–24 1932

David, L T The external expression and comparative dermal histology of hereditary hairlessness in mammals Z Zellforsch 14 616–719 1932

Deringer, M Spontaneous and induced tumors in haired and hairless strain hr mice J nat Cancer Inst 12 437–445, 1951

Dunn, T B Normal and pathologic anatomy of the reticular tissue in laboratory mice J nat Cancer Inst 14 1281–1433 1954

Dunn, T B & Deringer, M K Reticulum cell neoplasm, Type B, or the "Hodgkin's Like Lesion' of the mouse J nat Cancer Inst 40 771–821, 1968

Elgjo, K Epidermal cell population kinetics after a single application of some hyperplasia producing substances Europ J Cancer 3 519–530, 1968

Iversen, O H & Evensen, A Experimental skin carcinogenesis in mice Acta path microbiol scand , Suppl 156, 1962

Iversen, U & Iversen, O H Cycles of hair growth in hairless mice Acta path microbiol scand 69 50–62, 1967

Lignac, G O E Die Benzolleukämie bei Menschen und weissen Mäusen Krankheitsforsch 9 403–453, 1932

Lærum, O D Respiration and glycolysis in relation to cell population kinetics in mouse epidermis treated with carcinogenic or irritant substances. Europ J Cancer 8 239–253, 1972

Lærum, O D & Bjerknes, R Seasonal and individual metabolic variations of hairless mouse skin J invest Derm 58 284–290, 1972

Lærum, O D & Iversen, O H Reticuloses and epidermal tumors in hairless mice after topical skin applications of cantharidin and asiaticoside Cancer Res 32 1463–1469, 1972

Metcalf, D , Wiadrowski, M & Bradley, R Analysis of the role of the thymus in leukemia development with the use of thymectomy and thymus grafts Nat Cancer Inst Monogr 22 571–586, 1966

Peters, R L , Rabstein, L S , Spahn, G J , Madison, R M & Huebner, R J Incidence of spontaneous neoplasms in breeding and retired breeder BALB/cCr mice throughout the natural life span Int J Cancer 10 273–282, 1972 a

Peters R L , Hartley, J W , Spahn, G J , Rabstein L S , Whitmire, C E , Turner, H C & Huebner, R J Prevalence of the group specific (gs) antigen and infectious virus expressions of the murine C type RNA viruses during the life span of BALB/cCr mice Int J Cancer 10 283 289, 1972 b

Scheuplein, R J Permeability of the skin Physiol Rev 51 702 747, 1971

Acta path microbiol scand Section A 81, 64-70, 1973

AMINE-HANDLING PROPERTIES OF APUD-CELLS IN THE BRONCHIAL EPITHELIUM OF HUMAN FOETUSES AND IN THE EPITHELIUM OF THE MAIN BRONCHI OF HUMAN ADULTS

ESTHER HAGE

University Institute of Pathology, Odense Hospital

In bronchial epithelium of human *foetuses* cells containing fluorogenic amine were observed to take up amine precursors and to synthesize and store further amounts of amine Other cells apparently devoid of a cellular store of amine were capable of taking up *amine* precursors The possibility of distinguishing two cell types is discussed In human adults no aminecontaining cells were observed in the epithelium of the main bronchi However a few cells with a specific fluorescence were obtained after treatment with the precursor amino acid L DOPA

Common cytochemical characteristics of cells producing polypeptide hormones are amine precursor uptake and decarboxylation and accordingly, they have been given the name APUDcells (*Pearse* 1969) This is demonstrated by formaldehyde induced fluorescence of amines (*Falck et al* 1965) and therefore, the whole process has been described as APUD FIF (*Pearse et al* 1971)

Fluorescent cells were observed in the epithelium of the bronchi and bronchioles of newborn infants after treatment with gaseous formaldehyde (*Lauweryns et al* 1969) In the tracheo-bronchial epithelium of the mouse, no cellular amine fluorescence was observed, but cells capable of synthesizing and storing amine were demonstrated after administration of amine precursors (*Ericson et al* 1972) In the bronchial epithelium of human foetal lung cells containing fluorogenic amine as well as cells taking up amine precursors were found (*Hage* 1972)

The present investigation deals with the mono-amine mechanism of bronchial APUD cells of human foetuses and adults

Received 8 viii 72 Accepted 26 viii 72

Request for reprints should be addressed to Dr *E Hage* University Institute of Pathology Odense Hospital 5000 Odense Denmark

MATERIAL AND METHODS

The material comprised 18 human foetuses removed by Caesarean section in connection with legal abortion Crown rump length of the examined foetuses varied from 35 mm to 140 mm Measurements were made on unfixed foetuses held in a supine position The lungs were dissected out and small pieces of pulmonary tissues were incubated at 37° C in a continuously oxygenated Ty

rode s solution co ntaining various agents (*Hakan son et al* 1969)

The incubation media and incubation times used in six experimental groups are described in Table 1

After incubation the specimens were washed in co d (4° C) Tyrode s solution for 1 hr They were subsequently quenched to the temperature of li quid nitrogen freeze dried and exposed for 1–1½ hr at 80° C to gaseous formaldehyde (made from paraformaldehyde) for histochemical demonstration of mono amines The paraformaldehyde used in this reaction was standardized in an atmosphere of constant relative humidity between 50 70 per cent according to *Hamberger et al* (1964) Some pieces were not exposed to gaseous formaldehyde and served as control

Non incubated specimens were exposed to gase ous opthaldialdehyde (OPT) for demonstration of histamine (*Ehinger et al* 1968) All specimens were embedded in paraffin in vacuo serially sec tioned and mounted in Entellan

Fluorescence microscopy was carried out with a Leitz Orthoplan microscope using a mercury arc lamp (HBO W) with exciter filter BG 12/3 and barrier filter 530 nm For demonstration of hista mine exciter filter UG 1/1 and barrier filter 430 nm was used

Further incubation experiments were carried out with biopsies of the main bronchi of five human adults In these experiments only the system of *DOPA* and *DA* was used and group 4 was ex cluded

After fluorescence microscopy sections of group 1 and 2 were stained by alkaline diazonium tech nique with Fast black K (*Solcıa et al* 1969a),

TABLE 1 *Fluorescence Characteristics of APUD Cells in the Bronchial Epithelium of Human Foetuses*

Group no	Pretreatment mg/ml	Pretreatment hr	Treatment mg/ml	Treatment hr	Fluorescence intensity	Fluorescence colour	Number of cells	"Type 1 cells	Type cells
1					+ +	Greenish yellow	+	+	
2	*Pargyline* 0 08	¾			+ + (+)	Greenish yellow	+	+	
3			*L DOPA* 0 02	1½	+ + + +	Green	+ + +	+	+
			L 5 HTP 0 025	1½	+ + + +	Yellow	+ + +	+	+
4			*D DOPA* 0 20	1½	+ + (+)	Green	+ (+)	+	+
			D 5 HTP 0 025	1½	+ + (+)	Yellow	+ (+)	+	+
5	*Ro 4 4602* 0 075	¾	*L DOPA* 0 020	¾	+ +	Yellowish	+	+	+
					+	Greenish	+ (+)	+	+
			L-5 HTP 0 025	¾	+ +	Yellowish	+	+	+
					+	Yellowish	+ (+)	+	+
6			*DA* 0 04	1½	+ + (+)	Green	+ (+)	+	+
			5 HT 0 05	1½	+ + (+)	*Yellow*	+ (+)	+	+

DOPA 3 4 dihydroxyphenylalanine
5-HTP 5 hydroxytryptophan
DA Dopamine
5 HT 5 hydroxytryptamine
Pargyline Monoamine oxydase inhibitor
Ro 4-4602 Amino-acid decarboxylase inhibitor



Fig 1–4 Fluorescence photomicrographs of bronchial epithelium of human foetuses formaldehyde reaction (1 3) Non treated specimens (× 134) Small number of greenish yellow fluorescent cells (4) Pretreated with pargyline (× 140) The fluorescence intensity is slightly increased

lead haematoxylin (Pb H) (*Solcia et al* 1969b) and *Grimelius* argyrophilic silver technique (1968)

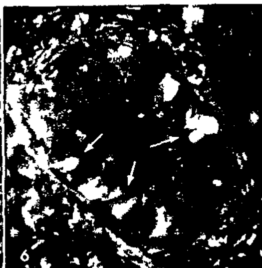
The reactivity of fluorescent cells to various staining methods was confirmed by photographing formaldehyde treated sections before and after subsequent staining in UV light and visible light respectively

RESULTS

By fluorescence microscopy of pulmonary tissue from human foetuses some scattered cells in the bronchial epithelium emitted a weak to moderate greenish yellow fluorescence upon formaldehyde treatment (group 1) The intensity of the fluorescence varied between different cells in the same bronchus The fluorescence was concentrated in the basal and paranuclear cytoplasm, giving the cell an oval or pyramidal configuration (Fig 1–3) By inhibiting mono amine oxydase with pargyline (Fig 4) the fluorescence intensity was slightly increased (group 2)

After incubation with L Dopa or L 5 HTP an increased number of epithelial cells were observed to emit a green or yellow fluore







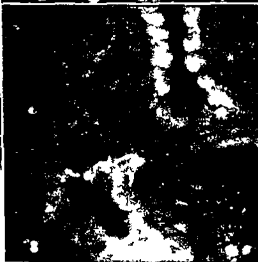


Fig 5–8 F... an foetuses formaldehyde reac... reen fluorescent cells (6) Pre... cells emit only a slight green ... (7) Incubated with L 5-HTP (× ...34) Large number of intense yellow fluorescent cells (8) Incubated with D 5 HTP (× 134) A few cells emit a yellow fluorescence of moderate intensity

scence respectively (group 3) The most intense fluorescence was observed in L-DOPA incubated specimens and frequently both nucleus and cytoplasm seemed to be fluorescent (Fig 5) In L-5 HTP incubated specimens the fluorescence of most cells was restricted to the cytoplasm (Fig 7) The largest number of cells was found at the division of the bronchi After incubation with the D isomer of DOPA or 5 HTP (group 4) the number of fluorescent cells was much smaller (Fig 8) and the fluorescence intensity of most cells was weaker than after incubation with the L-isomer (group 3) However compared to the untreated specimens of group 1 the number was slightly increased

Incubation with amine precursors after

5*

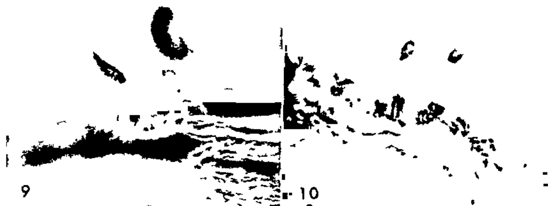


Fig 9 10 Fluorescence photomicrographs of epithelium of the main bronchi of human adults, formaldehyde reaction Incubated with L DOPA (× 134) A few cells emit an intense green fluorescence with an exclusively cytoplasmic localization

pretreatment with Ro 4 4602 (group 5) gave weaker fluorescence than incubation with precursors alone (group 3) This implies that amino acid which has been taken up by the cells is not retained in the cytoplasm unless it has been decarboxylated to corresponding amine according to *Håkanson et al* (1967) The normally occurring cellular store of amine was resistent to the action of Ro 4-4602 (Fig 6) Incubation with DA and 5-HT (group 6) produced only a weak fluorescence and the number of visible cells was much lower than after incubation with precursor amino acid

The mono amine mechanism of bronchial APUD cells was found to be independent of the age of the foetuses In foetuses more than 100 mm, a number of green fluorescent nerve terminals were observed These were located in a subepithelial position as well as in between epithelial cells

Using the histochemical OPT method by which to demonstrate histamine, mast cells were occasionally found in the interstitial tissues, but none were seen in the bronchial epithelium

The fluorescent cells of group 1 and 2 were stained brown black by Grimelius argyrophilic silver technique but failed to react to staining with diazonium and Pb H

In the epithelium of the main bronchi of human adults, no cells with formaldehyde induced fluorescence could be observed Neither did any fluorogenic amine content become visible after inhibition of the mono amine oxydase However, an intense green fluorescence with an exclusively cytoplasmic localization was observed in very few usually singly appearing cells after treatment with L DOPA (Fig 9–10) No specific cellular fluorescence was found after treatment with L DOPA or DA

In the bronchial epithelium of human adults no reaction could be obtained with the staining methods applied

DISCUSSION

In the bronchial epithelium of human foetuses amine content of some scattered cells could be demonstrated after formaldehyde treatment as a weak greenish yellow fluorescence The low intensity of the fluorescence corresponds to a low content of amine (*Ritzen* 1966) and this is in agreement with

the find ngs that the compound was too weakly reducing to give a positive diazo reaction

Compared to the number of amine containing cells, a greater number of cells were capable of taking up L-DOPA and L-5-HTP After treatment with L DOPA all cells showed intense green fluorescence an no cells with only a weak yellowish fluorescence could be identified This means, that amine containing cells have the capacity to take further amounts of amine precursors If cells of only *one type* exist, all have amine handling properties but only few contain detectable amounts of amine This could be due to the fact that the content is extremely low, or the turn over of the amines to non-fluorescent compounds is rapid However, pretreatment with mono-amine oxydase, which is active in the breakdown of the amine, increased the intensity of the fluorescence while the number of the cells re mained unchanged

If two cell types exist cells of *type 1* emit a weak fluorescence, reflecting a small amount of amine, and furthermore have the capacity of taking up amine precursors Cells of *type 2* are devoid of fluorogenic amines detectable by the formaldehyde and OPT technique, but have amine handling prop erties

Fluorogenic amine was not demonstrated in epithelial cells of the main bronchi of human adults, but after incubation with L DOPA a few fluorescent cells were observed Only a limited part of the bronchial system was studied however, and difference in the number of APUDcells in various parts of the bronchial tree of human adults cannot be excluded In the epithelium of bronchi and bronchioles of newborn infants *Lauweryns et al* (1969) observed cells with a content of fluorogenic amine A changing content of biogenic amines in APUDcells during development from newborn to adult has been showed in thyroid C cells of dogs (*Gershon et al* 1971) and in pancreatic A_2 and B cells of guineapigs (*Cegrell et al* 1968)

REFERENCES

1 *Cegrell, L & Falck, B* The development of monoamine containing cells in guinea pig pancreas Acta physiol scand 1968 Suppl 314

2 *Ehinger, B, Håkanson, R, Owman, C & Sporrong B* Histochemical demonstration of histamine in paraffin sections by fluorescence method Biochem Pharmacol *17* 1997–1998 1968

3 *Ericson, L F, Håkanson R, Larson B, Owman Ch & Sundler, F* Fluorescence and electron microscopy of amine-storing enterochromaffinlike cells in tracheal epithelium of mouse Z Zellforsch *124* 532–545 1972

4 *Falck B & Owman, Ch* A detailed methodological description of the fluorescence method for cellular demonstration of biogenic monoamine Acta Univ Lund Sectio II, No 7 1–23 1965

5 *Gershon, M, Belshaw, B & Nunez, E* Biochemical histochemical and ultrastructural studies of thyroid serotonin parafollicular and follicular cells during development in the dog Amer J Anat *132* 5–20, 1971

6 *Grimelius, L* The argyrophil reaction in islet cells of adult human pancreas studied with a new silver nitrate procedure Acta Societatis Medicorum Uppsaliensis 73 271–294 1968

7 *Hage, E* Endocrine cells in the bronchial mucosa of human foetuses Acta path microbiol scand A *80* 225–234 1972

8 *Hamberger, B Malmfors T & Sachs C* Standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines J Histochem Cytochem *13* 147 1965

9 *Håkanson, R Lilja B & Owman Ch* Properties of a new system of amine storing cells in the gastric mucosa of the rat Europ J Pharmocol *1* 188–199 1967

10 *Håkanson R Lilja B & Owman Ch* Cellular localization of histamine and mono amines in the gastric mucosa of man Histochemie *18* 74–86 1969

11 *Lauweryns J M & Peuskens J C* Argyrophil (kinin and amine producing?) cells in *18* 74–86 1969
human infant airway epithelium Life Sci (1) 8 577 585 1969

12 *Pearse A G E* The cytochemistry and ultrastructure of polypeptide hormone producing cells of the APUD series and the embryologic physiologic and pathologic implications of the concept J Histochem Cytochem *17* 303–313 1969

13 *Pearse, A G E & Polak J* Neural crest origin of the endocrine polypeptide (APUD)

cells of the gastrointestinal tract and pancreas Gut *12* 783-788 1971

14 *Ritzén, M* Quantitative fluorescence microspectrophotometry of catecholamine formaldehyde products Model experiments Exp Cell Res *44* 505 520 1966

15 *Solcia, E*, *Sampietro, R* & *Capella, C* Differential staining of catecholamines 5 hydroxytryptamine and related compounds in aldehyde fixed tissues Histochemie *17* 273-283, 1969a

16 *Solcia, E*, *Capella, C* & *Vassallo, G* Lead haematoxylin as a stain for endocrine cells Histochemie *20* 116-126, 1969b

Acta path microbiol scand Section A 81, 71–74, 1973

APPLICATION OF NYKA's METHOD FOR THE STAINING OF MYCOBACTERIA IN LEPROUS SKIN SECTIONS

AHMAD M MOHSEN* and WONDU ALEMAYEHU**

Armauer Hansen Research Institute (AHRI), affiliated with All Africa Leprosy Rehabilitation and Training Centre (ALERT), Addis Ababa, Ethiopia and The University of Bergen, Bergen, Norway Head Tore Godal, M D

Biopsy specimens from 20 leprosy patients have been studied. Adjacent sections were in each case both stained by the ordinary Ziehl Neelsen method and after oxidation with periodic acid (Nyka s method) Sections from tuberculoid, indeterminate and lepromatous lesions all revealed an increased number of acid fast bacilli after oxidation In ten examinations of tuberculoid and indeterminate cases the increase observed was in average four fold It is concluded that there is a considerable higher number of bacilli present in leprous tissue than revealed by the ordinary Ziehl Neelsen method

In leprosy there is a well established clinical and histopathological spectrum of disease manifestations (*Ridley* & *Jopling* 1966) The tuberculoid type represents the high resistant, often self limiting form, while the low resistant, diffuse infiltrative form, is found in lepromatous leprosy In tuberculoid leprosy few or no bacilli may be detected by the ordinary Ziehl-Neelsen (Z N) staining These small amounts of recognizable antigen, seem to be out of proportion to the strong immune response observed in such lesions

This could be explained in two ways

1 Considerable amounts of mycobacterial antigen is present in a soluble, non acid fast form
2 Only a proportion of the acid fast bacilli present in the lesions may be detected by the ordinary Z N staining

Nyka (1963, 1967), in his studies on the detection of *Mycobacterium tuberculosis* in tuberculous materials, found that bacilli which were completely decolorized if stained routinely with carbolfuchsin became fully acid fast after additional oxidation

In the present report, a modification of Nyka's method has been applied to the staining of leprous skin lesions The results show that by this method as compared to the ordinary Z N technique, a considerable higher number of acid-fast organisms may be detected in tuberculoid as well as lepromatous lesions

Received 6 iii.72 Accepted 4 vii 72

Requests for reprints should be addressed to dr T Godal, Armauer Hansen Research Institute (AHRI), P O Box 1005, Addis Ababa, Ethiopia

* Present address Department of Dermatology and Venereology, Faculty of Medicine, Alexandria University, Alexandria Egypt

** Medical student, Haile Selassie I University, Addis Ababa, Ethiopia

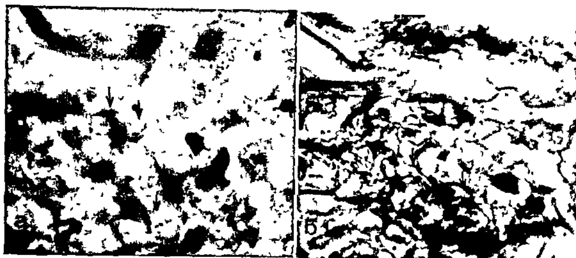


Fig 1 Adjacent sections from a lepromatous biopsy showing the same area (note the vessel cut through at the top) stained with a) ordinary Ziehl-Neelsen staining with a few bacilli (arrows) and b) modification of *Nyka's* method with numerous bacilli forming several agglomerations (globi) some of which are indicated by the arrows (× *1050*)

MATERIAL AND METHODS

Sections from 20 patients were used in this study Ten were from lepromatous patients, highly positive for *M leprae* The other 10 specimens were known to contain very few bacilli by the ordinary Z N method Two of them were diagnosed pathologically as indeterminate leprosy, and the other eight as tuberculoid leprosy *Nyka* (1967) used oxidizing agents (H_2O_2 or periodic acid) both during fixation and on sections before they were stained In our hands the fixation procedures used by Nyka made the specimens difficult to cut afterwards Moreover the by far most important step appeared to be the *oxidation of sections before staining Thus, in the* studies to be described, sections only have been oxidized

From the paraffin blocks serial sections were cut *five microns thick One of two adjacent sections* was stained by the ordinary Z N method as follows

After mounting, the section was left at 60°C for 1 hour and the paraffin removed by xylol 2 × 2 minutes and absolute alcohol 2 × 2 minutes The section was then rehydrated in water and left for 2 minutes in 70 per cent alcohol After exposure of warm carbolfuchsin for 10 minutes, the section was washed in water and decolourized in 0 5 per cent hydrochloric acid in 70 per cent alcohol until no more colour came off The section was again washed in water and counterstained with 0 5 per cent methylene blue for 1 minute

The other section was, after removal of paraffin and rehydration, left for four hours in a trough filled with a 5 per cent solution of periodic acid The section was *washed in tap water, rinsed in* distilled water and then placed in a trough filled *with carbolfuchsin prepared as follows Basic* fuchsin 1 gram, absolute alcohol 10 ml, distilled water 100 ml, phenol 5 6 grams The trough was incubated at 70°C for 30 minutes After being rinsed in tap water the section was decolourized in three changes of acid alcohol (70 per cent alcohol 100 ml, lactic acid 2 ml) Finally, it was washed in tap water counterstained lightly with haematoxylin, washed, blued in saturated lithium carbonate solution washed, dehydrated with acetone and mounted in Depex

Ten per cent periodic acid as used by *Nyka,* gave very little contrast between bacilli and background tissue We found after studies both involving variations in the period of incubation and the concentration of periodic acid the above procedure with 5 per cent periodic acid to give better results

RESULTS

In the cases of lepromatous leprosy, the present technique of oxidation showed a marked difference from the ordinary Z N staining The sections which were oxidized before staining showed significantly greater number, of bacilli (see Fig 1) and a higher proportion tended to stain solidly However, because of difficulties in counting the numerous bacilli in lepromatous tissue, no precise quantitations could be made In spite of the fact that the staining of *M leprae* was remarkable in uniformity and intensity, yet the

very sharp contrast was lacking because the pink colouration of the whole sections persisted in oxidized sections

In the ten non lepromatous specimens, the sections stained with the routine Z N stain showed very few bacilli These bacilli could easily be missed in the routine examination and were only identified by time consuming search of the whole section The number of bacilli found in the oxidized adjacent sections of these specimens are shown in Table 1

TABLE 1 *The Numbers of Bacilli Detected in Biopsy Specimens from Leprous Tissue by the Ordinary Ziehl Neelsen Method (Z N) as Compared to Those Detected in Adjacent Sections Stained by Our Modification of Nyka's Method*

Biopsy no	Diagnosis	No of bacilli per section	
		ordinary Z N	Nyka s method
43/70	Indeterminate	6	25
7/71	Indeterminate	2	9
77/71	Tuberculoid	5	22
110/71	Tuberculoid	6	23
12/71	Tuberculoid	31	162
196/71	Tuberculoid	20	113
158/71	Tuberculoid	23	110
203/71	Tuberculoid	69	182
224/71	Tuberculoid	48	157
228/71	Tuberculoid	54	170
Mean + SD		26 4±23 6	97 3±22 3

As shown in Table 1, a higher number (approximately four fold increase) of acid fast organisms was found in all the sections examined Assuming that the number of bacilli counted are normally distributed in both groups (as they seem to be), the difference is statistically highly significant ($P < 0.001$ by *t* test)

DISCUSSION

Several methods have been claimed to be superior to the Ziehl Neelsen method for the detection of acid fast organisms in leprous tissue (see *Wade* 1957) However to our knowledge, no quantitative estimation has been performed The present study shows that the number of acid fast organisms detected in a biopsy section from leprous tissue may be considerably increased by oxidation of the sections before staining In tuberculoid or indeterminate leprosy the increase was approximately four fold Thus, the ordinary Ziehl Neelsen method when applied to tissue sections, would seem to be relatively insensitive for detection of acid fast bacilli The strikingly low numbers of bacilli usually detected in tuberculoid leprosy may therefore, at least in part, be due to the staining method itself

It is interesting to note that the morphology of the bacilli also changed after oxidation A higher proportion of bacilli appeared to stain solidly As it is now well established that irregular stained bacilli are dead bacilli and solid ones only may be alive (see *Rees* 1969, *McRae & Shepard* 1971) this study indicates that not only the viability of the mycobacterium determines the staining quality Thus, only if carried out under standardized conditions the morphology of the bacilli may be used as reliable method for evaluating their viability

Because of the relatively poor contrast it would still not seem advisable without further modifications, to use Nyka s method as a routine method in leprosy

We wish to thank Dr *Dorothy R Samuel* for teaching us the pathology of leprosy and Mr *Kiros Ayenew* for skilled technical assistance

REFERENCES

McRae D H & Shepard C C Relationship between the staining quality of *Mycobacterium leprae* and infectivity for mice Infect and Immun *3* 116–120 1971

Nyka W Studies on *Mycobacterium tuberculosis* in lesions of the human lung A new method of staining tubercle bacilli in tissue sections Amer Rev Resp Dis *88* 670–679, 1963

Nyka W Method for staining both acid fast and chromophobic tubercle bacilli with carbolfuchsin J Bact *93* 1458–1460, 1967

Rees, R J W New prospects for the study of

leprosy in the laboratory Bull Wld Hlth Org *40* 785–800, 1969
Ridley, D S & *Jopling, W H* Classification of leprosy according to immunity A five group system Internat J of Leprosy *34* 255–273, 1966
Wade, H W A modification of the Fite formaldehyde (Fite I) method for staining acid fast bacilli in paraffin sections Stain Technol *32* 287–292, 1957

Acta path microbiol scand Section A 81, 75 78, 1973

THE IN VITRO MATURATION OF THE EMBRYONIC CHICKEN THYMUS

II *Development in Organ Cultures of Lymphocytes Responsive to Phytohaemagglutinin and Concanavalin A in vitro*

JAN F SALLSTROM and GUNNAR V ALM

Department of Histology, Institute of Human Anatomy, University of Uppsala, Uppsala Sweden

Thymic anlagen of 10 day old chick embryos were allowed to mature for 10 days in organ culture Lymphocyte suspensions were prepared from such anlagen with a mean yield of 2.85×10^5 cells per anlage If subcultivated, such lymphocytes were stimulated by the lymphocyte mitogens Phytohaemagglutinin (PHA) and Concanavalin A (Con A) These results suggest in particular that lymphocytes may acquire their reactivity to PHA and Con A in the thymic micro-environment In general the usefulness of the organ culture technique for the clarification of thymic functions is indicated

It is well documented that the thymus has an important role in the morphological and functional development of the lymphoid system (4, 14) This is mediated through an export of cells (11, 12, 13, 16, 17, 26) or the production of a humoral factor (5, 8, 9, 19, 24, 25) Most likely the thymus operates through both these mechanisms (21, 22)

The peripheral lymphoid cells of thymus deprived mice (6, 7, 23) and chickens (1, 10) demonstrate a decreased responsiveness to mitogens, such as Phytohaemagglutinin (PHA) Direct evidence that thymus dependent murine lymphocytes carrying θ antigens are responsive to PHA and Concanavalin A (Con A) has also been presented (3 20) Furthermore PHA reactive cells have been demonstrated in the embryonic human thymus earlier than in any other known locality (18) Taken together this strongly suggests, but does not prove that lymphocytes may acquire their responsiveness to PHA in the thymic micro-environment

Received 11 viii 72 Accepted 17 ix 72

In the present investigation this possibility was directly tested Thymic anlagen of 10 day old chick embryos were allowed to develop in organ culture for a 10 day-period (2) Thymic lymphocytes prepared from the lymphoid anlagen were subcultivated in the presence of PHA or Con A It was found that a fraction of the thymic cells responded to these mitogens This suggests that PHA and Con A responsive lymphocytes may develop within the thymus

MATERIAL AND METHODS

Embryos The White leghorn chick embryos were of the DeKalb Chix strain 161 (Hinseberg Hatchery Hinseberg Sweden) Details of the incubation procedures of the eggs have previously been described (2)

Organ culture technique The procurement of the thymic anlagen of the 10 day old chick embryos and the organ culture technique are described in detail elsewhere (2) In the present investigation the Waymouth's MB 752/1 tissue culture medium was supplemented with 10 per

cent chicken serum (CS) with or without further addition of 5 per cent embryo extract (EE_{50})

Preparation of thymic lymphocytes Thymic anlagen were harvested after 10 days in organ culture The two anlagen from each embryo were dissected free of the surrounding Spongostan gelatin foam and gently homogenized in small glass homogenizers in a 0 2 ml volume of a mixture of equal parts of Hanks balanced salt solution (HBSS) and Dulbeccos phosphate buffered saline (DPBS) supplemented by 5 per cent chicken serum The cell suspensions were transferred to 12 × 75 mm disposable polystyrene tubes (Falcon Plastics Oxnard Cal USA) and more of the HBSS DPBS CS solution was added Cell aggregates were allowed to sediment the supernatants were collected and centrifugated at 400 × g for 10 minutes The cells were resuspended in Waymouth s MB 752/1 tissue culture medium (Grand Island Biologicals Grand Island NY USA) supplemented with penicillin (100 U/ml) streptomycin (100 μg/ml) and 10 per cent heat inactivated chicken serum (56°C for 30 minutes) The cell concentration was adjusted to 2 5–5 × 10^6 cells per ml

Lymphocyte cultures Volumes of 0 1 ml cell suspension were cultured in 30 × 6 mm glass culture tubes at 37 5°C in a water saturated atmosphere of 5 per cent CO in air The culture time was 48 hours

Mitogens 1 Phytohaemagglutinin (Burroughs Wellcome Co London England) was first reconstituted with 5 ml 0 85 per cent NaCl per vial 10 μl of further dilutions was added per culture giving a final concentration of 0 3125 per cent or 1 25 per cent of the vial stock solution

2 Concanavalin A (Con A) was kindly provided by Dr D Campbell Pharmacia Uppsala Sweden The lyophilized product was dissolved in 0 85 per cent NaCl and sterilized by membrane filtration 10 μl was added per culture making the final Con A concentration 5 or 20 μg/ml medium

The incorporation of H^3 methyl thymidine The incorporation of H^3 methyl thymidine (H^3 TdR) was taken as an indicator of the rate of DNA synthesis and cell proliferation in the cultures After 48 hours 0 2 μc H^3 TdR spec activity 6 7 c/mM (New England Nuclear Boston Mass USA) in 25 μl 0 85 per cent NaCl was added per culture The cultures were harvested 4 hours later and frozen

Radioactivity measurements The culture tube contents were transferred to glass centrifuge tubes and extracted twice with cold 5 per cent (w/v) trichloro-acetic acid and absolute methanol respectively The final precipitates were each dissolved in 0 2 ml Soluene (Packard Instruments Co Downers Grove Ill USA) Permablend scintillator (Packard Instruments) containing 5 0 g PPO and 0 5 g bis MSB per 1000 ml toluene was added and the samples were counted in a liquid scintillation counter

The radioactivity measurements were recorded as counts per minute (cpm) per culture The change in the H^3 TdR incorporation caused by the mitogen was expressed for each embryo as the Stimulatory Index (SI) i e the ratio between the incorporation (cpm) in cultures with and without mitogen The Student s *t test was used for the* statistical evaluation

RESULTS

Yields of lymphocytes in organ cultured thymus The thymus anlagen of 10 day old embryos after 10 days in organ culture contained large numbers of lymphoid cells Table 1 demonstrates the number of lymphocytes obtained from cultures of thymic anlagen using a tissue culture medium supplemented with chicken *serum (CS) respectively* CS and embryo extract (EE_{50}) The mean yield per anlage was 2 64 × 10^6 lymphocytes with CS alone and 2 97 × 10^6 lymphocytes with CS + EE_{50}.

Lymphocyte reactivity to mitogens No differences could be demonstrated between the PHA or Con A reactivity of lymphocytes prepared from organ cultures with and without embryo extracts and the results with the two *supplements* are therefore considered together

Table 2 shows that in four separate experiments Phytohaemagglutinin (PHA) significantly increased the incorporation of H^3 TdR as demonstrated by the mean Stimulatory Index (SI) 1 88 ± 1 01 ($P < 0.001$) 23/30 embryos showed an SI greater than one

In three experiments Concanavalin A (Con A) increased the H^3 TdR incorporation with a mean SI of 2 23 ± 1 56 ($P < 0.025$) 10/12 embryos had an SI greater than one

DISCUSSION

The present investigation has demonstrated that the organ culture technique for the thymic anlagen of 10 day old chick embryos previously described (2) yields sufficiently large numbers of lymphocytes per thymic

TABLE 1 *Lymphocyte Yield per Thymic Anlage after 10 Days in Organ Culture*

Medium supplement*	No cell suspensions assayed	No anlagen per suspension		Lymphocyte yield per anlage	
		mean	range	mean	range
CS	24	3 38	1–16	$2\,64 \times 10^6$	$0\,69–4\,95 \times 10^6$
CS + EE_{50}	22	3 18	1–14	$2\,97 \times 10^6$	$1\,03\;6\,78 \times 10^6$

* CS indicates 10 per cent chicken serum
EE_{50} indicates 5 per cent embryo extract

TABLE 2 *Effect of PHA and Con A on the DNA Synthesis in Cultures of Embryonic Thymus Lymphocytes Mean Stimulatory Indices (S.I) ± S D* *

Experiment	PHA	Con A
1	1 81 ± 1 19 (11)**	
2	1 93 ± 0 90 (8)	3 23 ± 2 04 (4)
3	1 70 ± 0 48 (6)	2 21 ± 1 23 (4)
4	2 21 ± 1 37 (5)	1 27 ± 0 81 (4)
Total	1 88 ± 1 01 (30)	2 23 ± 1 56 (12)
Probability	< 0 001	< 0 025

* Stimulatory index The ratio between the H^3-thymidine incorporation in cultures with and without mitogen

** Figures within parenthesis are the number of embryos assayed

anlage to allow studies of their functional properties

Such lymphocytes were found to be reactive to the mitogens Phytohaemagglutinin (PHA) and to Concanavalin A (Con A) Even if the obtained responses were small, the findings suggest that PHA and Con A reactive lymphocytes develop in the *in vitro* grown isolated thymus Most likely these responsive cells originate from the small number of lymphoid stem cells present in the thymus of the 10 day-old chick embryo (15) at the initiation of the organ cultures Furthermore these results suggest that the embryonic chicken thymus shows both a morphological and functional maturation in organ culture, at least with respect to the lymphoid cells

In general the results of the present investigation suggest the potential usefulness of the organ culture technique for further studies of the role of the thymus in the development of the immunocompetent thymus dependent lymphocytes Such investigations are being undertaken

Addendum We have recently found (Sallstrom, J F and Alm, G V, to be published) that lymphocytes prepared from organ cultured thymic anlagen show much improved responses to PHA and Con A in serum-free RPMI 1640 tissue culture medium

Supported by the *Swedish Cancer Society* (Grant No *511-B71-01X) and Expressens Prenatalfond*
Miss *Marianne Eriksson* and Miss *Annsofi Holst* provided competent technical assistance

REFERENCES

1 *Alm, G V* In vitro studies of chicken lymphoid cells IV The effect of thymectomy on the antibody response in vivo and on the in

2

maturation of the embryonic chicken thymus I Development of an organ culture system Acta path microbiol scand Sect A *80* 778–784, 1972

3 *Blomgren, H & Svedmyr, E* Evidence for thymic dependence of PHA reactive cells in spleen and lymph nodes and independence in bone marrow J Immunol *106* 835–841, 1971

4 *Cooper, M D, Gabrielsen, A E & Good, R A* Role of the thymus and other central lymphoid tissues in immunological diseases. Ann Rev Med *18* 113–138, 1967

5 *Davies, A J S* The thymus humoral factor under scrutiny Agents and Actions *1* 1–7, 1969

6 *Doenhoff, M J, Davies, A J S, Leuchars, E & Wallis, V* The thymus and circulating lymphocytes of mice Proc Roy Soc London, Ser B *176* 69–85, 1970

7 *Doenhoff, M J & Davies, A J S* Reconstitution of the T-cell pool after irradiation of mice Cellular Immunol *2* 82–90, 1971

8 *Goldstein, A L, Asanuma, Y, Battisto, J R, Hardy, M A, Quint, J & White, A* Influence of thymosin on cell mediated and humoral immune responses in normal and in immunologically deficient mice J Immunol *104* 359 366, 1970

9 *Goldstein, A L, Guha, A, Howe, M L & White, A* Ontogenesis of cell mediated immunity in murine thymocytes and spleen cells and its acceleration by thymosin, a thymic hormone J Immunol *106* 773–780, 1971

10 *Greaves, M F, Roitt, I M & Rose, M E* Effect of bursectomy and thymectomy on the responses of chicken peripheral blood lymphocytes to phytohaemagglutinin Nature (London) *220* 293–295, 1968

11 *Harris, J E & Ford, C E* Cellular traffic of the thymus Experiments with chromosome markers Evidence from parabiosis for an afferent stream of cells Nature (London) *201* 884–887, 1964

12 *Hemmingsson, E J* Ontogenetic studies on lymphoid cell traffic in the chicken Acta Univ Upsal Abstracts Uppsal Dissert Med *112*, 1971

13 *Linna, T J & Stillstrom, J* Migration of cells from the thymus to the spleen in young guinea pigs Acta path microbiol scand *68* 465–475, 1966

14 *Miller, J F A P & Osoba, D* Current concepts of the immunological function of the thymus Physiol Rev *47* 437 520, 1967

15 *Moore, M A S & Owen, J J T* Experimental studies on the development of the thymus J Exp Med *126* 715–726, 1967

16 *Murray, R G & Woods, P A* Studies on the fate of the lymphocytes III The migration and metamorphosis of in situ labelled thymic lymphocytes Anat Rec *150* 113–128 1964

17 *Nossal, G J V* Studies on the rate of seeding of lymphocytes from the intact guinea pig thymus Ann N Y Acad Sci *120* 171–181, 1964

18 *Papiernik, M* Correlation of lymphocyte transformation and morphology in the human thymus Blood *36* 470–479, 1970

19 *Small, M & Trainin, N* Contribution of a thymic humoral factor to the development of an immunologically competent population from cells of mouse bone marrow J Exp Med *134* 786–800, 1971

20 *Stobo, J D, Rosenthal, A S & Paul, W E* Functional heterogeneity of murine lymphoid cells I Responsiveness to and surface binding of Concanavalin A and Phytohemagglutinin J Immunol *108* 1–17, 1972

21 *Stutman, O, Yunis, E J & Good, R A* Studies on thymus function I Cooperative effect of thymic function and lymphohemopoietic cells in restoration of neonatally thymectomized mice J Exp Med *132* 583-600, 1970

22 *Stutman, O, Yunis, E J & Good, R A* Studies on thymus function II Cooperative effect of newborn and embryonic hemopoietic liver cells with thymus function J Exp Med *132* 601–612, 1970

23 *Takiguchi, R, Adler W A & Smith, R J* Cellular recognition *in vitro* by mouse lymphocytes Effects of neonatal thymectomy and thymus graft restoration on alloantigen and PHA stimulation of whole and gradient separated subpopulations of spleen cells J Exp Med *133* 63–80, 1971

24 *Trainin, N, Small, M & Globerson A* Immunocompetence of spleen cells from neonatally thymectomized mice conferred *in vitro* by a syngeneic thymus extract J Exp Med *130* 765 775, 1969

25 *Trainin, N & Small, M* Studies on some physicochemical properties of a thymus humoral factor conferring immunocompetence on lymphoid cells J Exp Med *132* 885–897, 1970

26 *Weissman, I L* Thymus cell migration J Exp Med *126* 291 304, 1967

Acta path microbiol scand Section A 81, 79–84, 1973

MIGRATION OF HAEMOPOIETIC CELLS FROM THE YOLK SAC TO THE THYMUS AND THE BURSA OF FABRICIUS IN THE CHICK EMBRYO

Erik J Hemmingsson and Gunnar V Alm

Department of Histology, University of Uppsala, Uppsala, Sweden

The haemopoietic cells of the yolk sac wall of 16-day-old chick embryos were locally labelled with ³H thymidine *in situ* Appropriate controls for the isotope leaking to other parts of the embryo during the local labelling procedure were performed With autoradiographic technique heavily labelled haemopoietic cells derived from the yolk sac were demonstrated in the thymus and in the bursa of Fabricius 24 hours later These results directly support the view that the haemopoitic areas of the yolk sac is at least one origin of the lymphoid precursor cells entering the embryonic chick thymus and bursa of Fabricius

The origin of the precursor cells for the lymphoid cell populations of the thymus in mammals and the chicken and the bursa of Fabricius in the chicken has been much debated (10)

On the basis of morphological studies of the early development of these central lymphoid organs two main theories were advanced Thus the lymphoid cells or their precursors were either thought to derive directly from the epithelial cells of the organ anlagen (1 2, 3) or from lymphoid precursor cells of mesenchymal origin migrating into the epithelial anlagen (7, 8, 17)

The work of *Auerbach et al* (4, 5) appeared to support a direct transformation of thymus epithelial cells into lymphoid cells More recent findings in contrast suggest that blood borne lymphoid precursor cells migrate into both the embryonic thymus and the bursa of *Fabricius* (14, 15, 16) Furthermore these immigrating precusor cells appear essential for the subsequent lymphoid development of at least the thymus (18)

Direct evidence relative to the origin of these precursor cells has been presented Thus, significant numbers of transfused embryonic bone marrow, spleen and yolk sac cells, previously labelled *in vitro* with ³H-thymidine, were traced both to the thymus and the bursa of Fabricius of the recipient chick embryos (12 13) However, transfusion experiments may not necessarily reflect the normal cell migration streams of the intact embryo

In the present investigation we decided to directly test whether haemopoietic cells of the yolk sac migrate to the thymus and to the bursa of Fabricius of the chick embryo Consequently the haemopoietic foci of the yolk sac of 16 day-old embryos were labelled locally *in situ* by the injection of ³H thymidine into the yolk sac wall As much of the ad

Received 30 viii 72 Accepted 22 ix 72

Requests for reprints should be addressed to Dr Erik J Hemmingsson Department of Histology, University of Uppsala Vastra Agatan 26 A S-752 20 Uppsala 1 Sweden

ministered isotope immediately leaks into the blood stream and labels tissues outside the yolk sac appropriate leakage controls were performed These were yolk sac labelled embryos sacrificed one hour after labelling, as well as embryos receiving an intravenous injection of the isotope

Examinations of the autoradiographs of the histological sections of the thymus and the bursa of Fabricius 24 hours after labelling of the yolk sac revealed a migration of heavily labelled yolk sac cells to both these organs

MATERIALS AND METHODS

Animals Fertilized eggs of the outbred White leghorn De Kalb Chix strain 161 (Hinseberg Hatchery, Hinseberg, Sweden) were incubated in a Funki type I automatic egg incubator (Funki Hammerum, Denmark) at 38° C and 55 per cent relative humidity with rotation every hour

Labelling On the 16th day of incubation, 12 chick embryos were labelled in the yolk sac wall by direct injection of ^{3}H methyl thymidine, spec act 6 7 Ci/mM (New England Nuclear Corp, Boston, Mass, U S A) Nine embryos received the isotope directly into one of the veins of the chorio allantoic membrane The ^{3}H methyl thymidine stock solution contained 250 μCi per 0 5 ml of sterile water and was further diluted with sterile physiological saline to a concentration of 30 μCi/ml Each animal received 50 μl (1 5 μCi) of the isotope solution either into the wall of the yolk sac or intravenously The isotope dose corresponded to approximately 0 07 μCi/g body weight

For local labelling of the cells of the yolk sac wall, a small hole (approximately 10×10 mm) was first cut in the egg shell The shell was removed and an incision was made in the chorio allantoic membrane The yolk sac was carefully grasped with a pair of forceps and part of it pulled out through the hole A microlitre syringe (Hamilton Comp Whittier Calif, U S A) with a fine gauge needle (external diameter 0 30 mm) was used to administer the ^{3}H methyl thymidine The needle was inserted in the yolk sac close to the forceps and the isotope was deposited in the yolk sac wall in three directions from the same point of insertion The yolk sac was then replaced in the egg and the hole covered with a piece of Parafilm® (Marathon Products Neenah Wisc U S A) The time for the whole labelling procedure did not exceed 3 minutes per animal

Dissection procedure One hour after labelling 4 embryos labelled locally in the yolk sac were killed by decapitation Twenty four hours after labelling, the remaining 8 embryos labelled in the yolk sac and 9 intravenously labelled embryos were sacrificed in the same way The following organs were sampled a 20×20 mm area of the yolk sac wall at the site of the ^{3}H thymidine injection the bursa of Fabricius, the thymus and a 10 mm piece of duodenum

Histological and autoradiographic technique The tissue specimens were immediately placed in 4 per cent carbonate buffered formalin for subsequent ethanol dehydration and xylene clearing according to standard methods All specimens were then embedded in paraffin wax and 5 micron sections were cut

Autoradiographs of these sections were prepared with the liquid emulsion technique (16) using NTB–2 emulsion (Eastman Kodak Co Rochester, N Y, U S A) The slides were exposed for 21 days in light proof boxes and subsequently developed in Kodak D 19 B for 2 minutes and fixed in Kodak F 24 for 10 minutes The sections were stained through the emulsion with methyl green and pyronine (MGP) A more detailed description of the MGP staining will be given elsewhere (Brenning T to be published)

Autoradiography and the transport of cells The technique used for labelling of the cells in the wall of the yolk sac can result in the appearance of labelled cells in other organs in two ways This can occur firstly as the result of migration of locally labelled cells or their breakdown products and secondly as a consequence of direct leakage of ^{3}H thymidine from the local site of injection into the vascular system Information about the leakage labelling of cells outside the locally labelled yolk sac was obtained in two different ways One group of animals labelled locally in the yolk sac wall were sacrificed one hour after the labelling that is after all the ^{3}H thymidine administered had been incorporated into proliferating cells (6) but before any significant number of labelled cells had left the yolk sac This control group was therefore supposed to give information about the amount of ^{3}H thymidine leaking at the local labelling injection Control animals injected intravenously with the same dose of isotope as that used for the yolk sac labelled animals and sacrificed at the same time as these after 24 hours were used to obtain an estimate of the maximal possible leakage labelling of cells in different organs

Evaluation of the autoradiographs The autoradiographs prepared from the sections of the organs of the animals of the three experimental groups were evaluated in the following manner

First grain counts were performed on all cells with more than 4 grains per cell in a total of 25 visual fields (magnification 1000 ×) One section from each organ of the animals in the yolk sac labelled 1 hour group and the intravenously labelled 24 hour group was examined The results were summarized and compared for the two groups

with respect to the maximal grain counts recorded in the thymus, the intrafollicular and interfollicular areas of the bursa of Fabricius and the mucosa and epithelium of the duodenum (Table 1)

Then one entire section from each organ of the intravenously labelled control animals was scanned for the presence of cells with more than 9 grains The observed maximal grain count per cell for each organ and the total number of cells with more than 9 grains were recorded (Table 1)

Finally one whole section of each organ of the yolk sac labelled animals sacrificed 24 hours after labelling was screened for the presence of cells with grain counts higher than the maximal grain counts found in the intravenously labelled control animals The location of these cells and their grain counts were recorded (Table 2)

RESULTS

Local labelling of the yolk sac wall Only a small part of the yolk sac was labelled locally with the ³H thymidine In the locally labelled area the haemopoietic foci contained many heavily labelled cells with different morphological characteristics (Fig 1) A



Fig 1 Autoradiograph showing h l c in the haemopoietic foci 1 hour after local *in situ* labelling of the wall of the yolk sac with ³H thymidine on the 16th day of incubation (Methylgreen and pyronine 1000 ×)

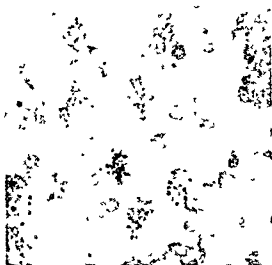


Fig 2 Autoradiograph of heavily labelled yolk sac derived cell in the thymic medulla 24 hours after local *in situ* labelling with ³H thymidine of the wall of the yolk sac in chick embryo on the 16th day of incubation (Methylgreen and pyronine, 1000 ×)

large proportion of these were immature haemopoietic cells with stem cell morphology No cell damage caused by the local injection of ³H-thymidine was seen by light microscopy

Estimation of the maximal grain counts in the control groups The maximal grain counts estimated by counting the grains over all cells with more than 4 grains in 25 visual fields (1000 × magnification) in one section of each organ from each of the animals of the yolk sac labelled 1 hour group and the intravenously labelled control group are given in Table 1 The maximal grain counts over the cells of the thymus the bursa of Fabricius (both intra- and interfollicular) as well as the duodenum were always as high or higher in the latter group Also the total number of labelled cells with more than 4 grains was higher in the intravenously labelled 24 hour group

Because of these findings the intravenously labelled control group was considered as the control of choice The control material in this group was increased by counting all cells with more than 9 grains in one whole section

TABLE 1 *Maximal Grain Counts Found over Cells in Different Tissues in the Control Animals*

Labelling	Number of animals	Thymus	Organ: Bursa of Fabricius interfollicular	Bursa of Fabricius intrafollicular	Duodenum
local labelling of the yolk sac, sacrificed 1 h later	4*	8 (28)§			14 (73)
	2*		12 (60)	7 (2)	
intravenous labelling, sacrificed 24 h later	9*	8 (32)	14 (270)	12 (66)	14 (391)
	9†	10 (1)	18 (18)	14 (4)	14 (3)

* Based on counting of all cells in 25 visual fields (magnification 1000 ×) with more than 4 grains in one histological section of the thymus, the bursa of Fabricius and the duodenum in each animal
§ Values in parenthesis represent total number of cells with more than 4 grains or more than 9 grains
† Based on counting of all cells with more than 9 grains in one histological section from the thymus and the bursa of Fabricius and 4 sections of the duodenum of each animal

TABLE 2 *Frequency and Localization of Heavily Labelled Yolk Sac Derived Cells (h l c) in Different Organs 24 Hours after* in situ *Labelling of the Cells of the Yolk Sac Wall with ³H Thymidine**

Organ	Number of animals	Lower limit for h l c (*grains/cell*)	Number of h l c found: Total no of h l c	No of h l c with > 20 grains/cell
Thymus	8			
cortex		10	41	18
medulla		10	27	14
interfollicular		10	17	9
Bursa of Fabricius	7			
intrafollicular		14	11	7
interfollicular		18	19	17
Duodenum	8	14	0	0

* One whole section from each of the organs of the yolk sac labelled animals was screened for the presence of heavily labelled yolk sac derived cells In the screening, only pyroninophilic cells with stem cell morphology were examined

from each organ from all animals The maximal grain counts per cell found in this way for the different tissues and the total number of cells with more than 9 grains are shown in Table 1 The maximal grain counts was for the thymus 10 grains, for interfollicular cells of the bursa of Fabricius 18 grains and for intrafollicular cells 14 grains and finally for the cells of the duodenal mucosa and epithelium 14 grains

Cells with grain counts higher than these figures in the yolk sac labelled 24 hour group were considered as cells immigrating from the locally labelled yolk sac They were designated heavily labelled cells (h l c) Only those h l c which were pyroninophilic were considered in this investigation

Migration of h l c from the locally labelled yolk sac One whole section of each of the organs of the yolk sac labelled 24 hour animals were screened for the presence of pyroninophilic h l c The results are given in Table 2 In the thymus, 85 h l c were found in a total of 8 sections from the same number of ani-

mals Out of these, 41 were located in the thymic cortex, 27 in the medulla and 17 in the stroma between the thymic lobules In seven sections from the bursa of Fabricius representing the same number of animals, a total of 30 h l c were found Out of these, 11 were located in the bursal follicles and 19 between the follicles No h l c could be demonstrated in the sections of the duodenum

A significant portion of the h l c, both in the thymus and the bursa of Fabricius of the locally labelled animals exhibited grain counts of 20 or more per cell (Table 2)

DISCUSSION

The results of the present investigation show that DNA synthesizing haemopoietic cells of the yolk sac wall were successfully locally labelled after the local application of ^{3}H-thymidine The injection of the ^{3}H thymidine into the yolk sac wall also resulted in labelling of cells outside the yolk sac by the leaking isotope Controls for this leakage labelling were locally labelled embryos sacrificed 1 hour after the administration of the isotope Within this time leaking isotope should be incorporated by cells outside the yolk sac As a further control embryos were labelled intravenously with the same isotope dose as that used for local labelling of the yolk sac wall and sacrificed 24 hours later In the latter control, the isotope may also label haemopoietic cells outside the site of the local labelling and such cells may within the 24 hour period migrate to the thymus and the bursa of Fabricius the target organs of this study

These leakage controls demonstrated that the leakage labelling was considerable Both the maximal grain counts and the number of labelled cells were higher in the intravenously labelled control animals This group should therefore be the appropriate control although it may still be unfavourable The maximal grain counts per cell possibly caused by the leaking isotope was therefore determined in the intravenously 24 hour leakage control group In the thymus and the bursa of Fabricius of the yolk sac labelled embryos sacrificed after 24 hours, any cell with grain counts higher than that of the controls were considered to be immigrants derived from the locally labelled cell population of the yolk sac wall The fact that the duodenal mucosa and epithelium of the locally labelled animals presented no increase in the number of heavily labelled cells attests to the validity of this reasoning

Significant numbers of heavily labelled yolk sac derived immigrants were found both in the cortex and the medulla of the thymus and in the bursa of Fabricius 24 hours after labelling Such heavily labelled cells were also found outside the lymphoid tissue proper in these organs These immigrant cells may be the precursor cells for the thymic and bursal lymphoid cells The results therefore support the hypothesis that the yolk sac is the origin of at least some of the cells entering the embryonic thymic and bursal anlagen (12–15)

Injection of *in vitro* ^{3}H thymidine labelled cells from the yolk sac and other embryonic haemopoietic organs into chick embryos resulted in an accumulation of labelled cells in the thymus and the bursa of Fabricius (12, 13) These results suggested that such cells have the capacity to home in these organs, but do not prove that they do this in the intact animal The technique of local labelling used in the present investigation presumably reflects more accurately the normal cell traffic patterns

As the yolk sac is a major producer and exporter of erythrocytes (11), the possibility must be entertained that the labelled immigrants in the bursa of Fabricius and the thymus are erythrocytes The fact that the heavily labelled immigrants morphologically did not resemble typical erythrocytes and furthermore that only such labelled cells as were pyroninophilic were considered as probable yolk sac derived cells speak against this possibility

The results of this investigation therefore demonstrate that the local labelling technique may be used to demonstrate a traffic of cells from the yolk sac to the thymus and the bursa of Fabricius Although the number of detect

6*

able immigrants in these organs was low it should be emphasized that only a small part of the yolk sac wall is labelled by the local injection of isotope Furthermore the only cells in this area to be labelled were those which were in the S phase during the isotope administration Such heavily labelled cells emigrating to the thymus and the bursa of Fabricius may also divide in these organs resulting in a dilution of the number of grains per cell Therefore the demonstrated traffic of cells in this investigation represents a minimum and may in reality be considerably larger than observed

---

The expert technical assistance of Miss *Lili Anne Eriksson* is gratefully acknowledged

This investigation was supported by grants from the *Swedish Cancer Society* (511 B71 O1X) the *Swedish Medical Research Council* (K72 12X 33 09) the *Expressens Prenatalfond* and *Allèns Stiftelse*

REFERENCES

1 *Ackermann G A* Electrone microscopy of the bursa of the embryon c chick with particular reference to the lymphoepithelial nodules J Cell Biol *13* 127 146 1962

2 *Ackermann G A* The lymphocyte its morphology and embryological orig n In The lymphocyte in immunology and haemopoiesis ed Yoffey J M p 11 30 Edward Arnold Ltd London 1967

3 *Ackermann G A* & *Knouff R A* The epithel al or gin of the lymphocyte in the thymus of the embryonic Hamster Anat Res *152* 35–54 1965

4 *Auerbach R* Experimental analys s of the orig n of cell types in the development of the mouse thymus Devel B ol *3* 336 354 1961

5 *Ball W D* & *Auerbach R* In vitro formation of lymphocytes from embryonic thymus Exp Cell Res *20* 245 247 1960

6 *Cleaver J E* Thymidine metabol sm and cell kinet cs p 58 North Holland Publ sh ng Co Amsterdam London 1967

7 *Hammar J A* Zur groberen Morpholog e und Morphologen e der Menschenthymus Anat Hefte *43* 201 242 1911

8 *Jolly J* La bourse de Fabr cius et les organes lympho ep théliaux Arch d Anat Micr *16* 363 547 1915

9 *Kopriwa B M* & *Leblond C P* Improvements in the coating technique of rad oautography J Histochem Cytochem *10* 269 284 1962

10 *Metcalf D* & *Moore M A S* Haemopo et c Cells p 224 261 North Holland Publ sh ng Co Amsterdam London 1971

11 *Metcalf D* & *Moore M A S* Haemopo etic Cells p 173 188 North Holland Publ sh ng Co Amsterdam London 1971

12 *Metcalf D* & *Moore M A S* Haemopo et c Cells p 238 240 North Holland Publ sh ing Co Amsterdam London 1971

13 *Metcalf D* & *Moore M A S* Haemopo etic Cells p 251 255 North Holland Publ sh ng Co Amsterdam London 1971

14 *Moore M A S* & *Owen J J T* Chromosome marker stud es on the development of the haemopo etic system in the ch ck embryo Nature *208* 956 and 989 990 1965

15 *Moore M A S* & *Owen J J T* Experimental studies on the development of the thymus J Exp Med *126* 715 726 1967

16 *Moore M A S* & *Owen J J T* Chromosome marker stud es in the irrad ated ch ck embryo Nature *215* 1081 1082 1967

17 *Ortega L G* *Kattine A A* & *Spurlock B O* Lympho ep thelial interact on in the developing bursa of Fabric us Fed Proc *24* 160 1965

18 *Owen J J T* & *Ritter M A* Tissue interaction in the development of thymus lymphocytes J Exp Med *129* 431–437 1969

Acta path microbiol scand Section A 81, 85–90, 1973

THE SPECIFICITY OF THE CHROMOSOMAL ABNORMALITIES IN HUMAN COLONIC POLYPS

A Cytogenetic Study of Multiple Polyps in a Case of Gardner's Syndrome

JOACHIM MARK, FELIX MITELMAN, HANS DENCKER, CLAES NORRYD and KARL-GORAN TRANBERG

Department of Pathology, Huddinge Hospital, and Departments of Medicine and Surgery, University of Lund, Sweden

The chromosomes were studied in 7 polyps of the large bowel in a case of Gardner's syndrome All tumours were histologically non invasive Four polyps had a normal diploid stemline and showed a predominantly hyperdiploid chromosome variation Three polyps had heteroploid stemlines two were pseudodiploid and one was hyperdiploid The chromosomal difference between polyps of different size was merely quantitative The same non random karyotypic pattern was found in all polyps irrespective of size and histological grading

Cytogenetic studies of premalignant lesions are of crucial importance for the understanding of the role of chromosomes in the development of the abnormal cell populations that usually characterize an invasive neoplasm Mainly for technical reasons, however knowledge in this field is very restricted Thus, precancerous lesions in man have been studied in detail only in the cervix uteri (see *Granberg* 1971) Information about comparable conditions at other sites is scanty or completely lacking (*Sandberg* & *Hossfeld* 1970) This applies also to polyps of the large bowel, in spite of their commonplace nature

The obvious need for further information of polyps of the large bowel prompted the present study which deals with the chromosomal observations in 7 different polyps from a patient with Gardner's syndrome This syndrome (*Gardner* & *Richards* 1953) follows an autosomal dominant pattern and is characterized by multiple colonic polyps with osteomas and various cutaneous and subcutaneous lesions The present case offered an excellent opportunity to study many polyps in their earliest stages of development

Received 8 ix.72 Accepted 12 ix.72

MATERIAL AND METHODS

The patient was a 36 year-old female, member of a family described elsewhere (*Alm et al* 1972) with Gardner's syndrome Previous histological examination of biopsy specimens from several colonic polyps had demonstrated atypia of varying degrees of severity Since 4 out of 7 members of the family had developed carcinoma of the large bowel a prophylactic colectomy was performed The entire colon was obtained from the operating room within a few minutes after operation Macroscopically the colon was crowded with polyps varying in size from about 1 mm in diameter to large pedunculated polyps the largest being 1 8 cm in diameter

Five small polyps, less than 4 mm in diameter

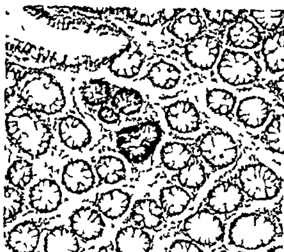


Fig 1 Histological picture of the small polyps the areas with atypia confined to isolated ducts Haematoxylin and eosin × 200



Fig 2 Histological picture of the large polyps widespread areas with severe atypia Haematoxyl n and eosin × 200

and 5 large ones 1 4 1 8 cm in diameter were subjected to chromosome analyses Chromosome preparations of good qual ty were obtained from 4 of the former polyp types Nos 1–4 and from 3 of the latter Nos 5 7 In the small polyps about half the tumour was used the remainder being used for histological exam nation In the larger polyps adjacent tumour tissue was selected for the histological examination Histologically all polyps showed areas with atypia In the small ones this was mostly confined to isolated ducts (Fig 1), whereas all large polyps had areas with severe atypia i e pleomorphic cells and sometimes highly irregular glands (Fig 2) All tumours were non invasive

The chromosomes of all polyps were stud ed in direct fixations by the same method as described in detail by *Mark* (1969) In each polyp the chromosomes of 18 50 metaphases were counted and 3 11 cells were karyotyped in detail Karyotype analyses were made by photography Chromosome band ng however was not feas ble

RESULTS

The chromosome counts in the 7 polyps stud ied are presented in Table 1 It is seen that the normal diploid chromosome number dominated in all polyps except one (No 4) which had a bimodal distribution Deviating cells, mostly hyperdiploid ones were observed in all polyps The spread however was fair ly restricted, in the whole material 96 per cent of the counts were found within the range 46 ± 2 Only 2 polyploid cells both in the tetraploid region, were observed

Table 2 presents the karyotypic findings in the 7 polyps as gains or losses in relation to the normal diploid female karyotype Out of the 4 small polyps (Nos 1–4), 2 had a normal diploid stemline 1 was pseudodiploid and 1 had a hyperdiploid stemline Out of the 3 large polyps (Nos 5–7) 2 had a normal diploid stemline and 1 was pseudodiploid It appears from the table that all abnormal stemlines as well as all variant cells except 3 displayed changes in only 2 chromosome groups i e C and D These chromosome types were involved also in 2 of the 3 cells which in addition displayed other changes as well It should be noted that the chromosome group D was predominantly seen as extra whereas chromosome type C was predom inantly missing Thus all hyperdiploid and pseudodiploid cells except 1 showed 1 2 ex tra D chromosomes and 1 3 C chromosomes were missing in all hypodiploid cells It should also be noted that the 2 pseudodiploid polyps had identical karyotype $-1C$ $+1D$ (Fig 3 a)

Markers were found in a single cell in polyp No 1 This tetraploid cell differed from a normal doubled cell only by the gain

TABLE 1 *Chromosome Counts in 7 Polyps of the Large Bowel*

Polyp no	Chromosome number												Total of cells
	41	42	43	44	45	46	47	48	49	50	51	±4x	
1						26	1	1			1	1	30
2			1	2	1	18	1	1	1			1	26
3		1		1		18	1						21
4						8	1	9					18
5	1		1	2	1	35							40
6					2	25	5	1					33
7					1	34	9	5	1				50

TABLE 2 *Karyotypic Findings in 7 Polyps of the Large Bowel*

Polyp no	Chromosome number S = stemline	Karyotype as related to the normal diploid karyotype	Number of cells
1	S = 46	Normal	9
	47	+1D	1
	94*	+2t markers	1
2	43	—2C, —1D	1
	44	—1C, —1D	2
	45	—1C	1
	S = 46	Normal	4
	49	+1C, +2D	1
3	42	—2C, —1D, —1G	1
	44	—1C, —1D	1
	S = 46	—1C +1D	1
4	47	—1C, +2D	1
	S = 48	+2D	2
5	41	—1A3 —2C, —1D, —1E18	1
	43	—3C	1
	44	—2C	2
	45	—1C	1
	S = 46	Normal	1
6	45	—1C	2
	S = 46	Normal	4
	47	+1D	3
7	46	Normal	2
	S = 46	—1C, +1D	3
	48	+2D	1
	48	+2C	1

* In relation to the tetraploid female karyotype

of 2 markers with terminal centromere One of these was half as big as a G chromosome, the other had a size intermediate between the chromosomes of groups D and G The latter marker was similar to the one earlier observed in colonic carcinoma (*Lubs & Kotler* 1967) In this context, it is also of interest that, in the present study, one D

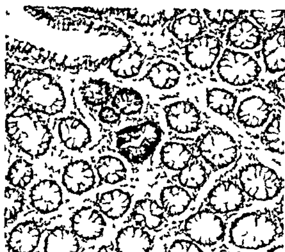


Fig 1 Histological picture of the small polyps the areas with atypia confined to isolated ducts Haematoxylin and eosin × 200



Fig 2 Histological picture of the large polyps widespread areas with severe atypia Haematoxylin and eosin × 200

and 5 large ones 1 4 1 8 cm in diameter were subjected to chromosome analyses Chromosome preparations of good quality were obtained from 4 of the former polyp types Nos 1–4 and from 3 of the latter, Nos 5 7 In the small polyps about half the tumour was used the remainder being used for histological examination In the larger polyps adjacent tumour tissue was selected for the histolog cal examination H stolog cally all polyps showed areas with atypia In the small ones this was mostly confined to isolated ducts (Fig 1), whereas all large polyps had areas with severe atypia i e pleomorphic cells and sometimes highly irregular glands (Fig 2) All tumours were non invasive

The chromosomes of all polyps were stud ed in d rect fixations by tl e same method as descr bed in detail by *Mark* (1969) In each polyp tl e chromosomes of 18 50 metaphases were counted and 3 11 cells were karyotyped in detail Karyotype analyses were made by photography Chromosome banding l owever was not feasible

RESULTS

The chromosome counts in the 7 polyps stud ied are presented in Table 1 It is seen that the normal diploid chromosome number dominated in all polyps except one (No 4) which had a bimodal distribution Deviating cells, mostly hyperdiploid ones were observed in all polyps The spread however, was fair ly restricted, in the whole material 96 per cent of the counts were found within the range 46 ± 2 Only 2 polyploid cells both in the tetraploid region were observed

Table 2 presents the karyotypic findings in the 7 polyps as gains or losses in relation to the normal diploid female karyotype Out of the 4 small polyps (Nos 1–4), 2 had a normal diploid stemline 1 was pseudodiplo d and 1 had a hyperdiploid stemline Out of the 3 large polyps (Nos 5–7), 2 had a normal diploid stemline and 1 was pseudodiploid It appears from the table that all abnormal stemlines as well as all variant cells except 3 displayed changes in only 2 chromosome groups i e C and D These chromosome types were involved also in 2 of the 3 cells which, in addition displayed other changes as well It should be noted that the chromosome group D was predominantly seen as extra whereas chromosome type C was predom inantly missing Thus all hyperdiploid and pseudodiploid cells except 1 showed 1–2 ex tra D chromosomes and 1–3 C chromosomes were missing in all hypodiploid cells It should also be noted that the 2 pseudodiploid polyps had identical karyotype —1C +1D (Fig 3 a)

Markers were found in a single cell in polyp No 1 This tetraploid cell differed from a normal doubled cell only by the gain

some groups C and D, the former as losses and the latter as gains Other chromosome groups were rarely involved These findings gained in importance when all materials of colonic polyps from the literature were reviewed (*Enterline* & *Arvan* 1967, *Lubs* & *Kotler* 1967, *Baker* & *Atkin* 1970), and it was revealed in all of these that the C and D chromosomes were those most often undergoing variation in number Thus, out of 16 polyps of the large bowel with deviating cells and in which karyotype analyses were recorded, 13 had stemlines or variant cells with involvement of C and/or D chromosomes This selective involvement is a clear indication of a non-random karyotypic pattern in colonic polyps

It is well known that carcinomas of the large bowel have modal chromosome numbers which tend to favour the hypertriploid region (*Yamada et al* 1966, *Lubs* & *Kotler* 1967, *Miles* 1967, *Atkin* 1970) It would be of great interest to compare the chromosome patterns of the polyps to those of carcinomas of the large bowel Unfortunately, this is at present impossible because of lack of information It should be pointed out, however, that at least some of the carcinomas studied have shown undisputable deviations in groups C and D, as in the present study, although too few have been karyotyped in detail to permit any comparison

Heredity has for a long time been known to play a role in the genesis of intestinal polyposis of the large bowel (*Cripps* 1882 *McKusick* 1962) It is likely, but thus far purely speculative, that ingested carcinogens are contributary The aetiology of colonic carcinoma can thus be considered to involve both an intrinsic or congenital factor and an extrinsic, superimposed carcinogenic influence This idea is of interest in view of recent results from virus induced and chemically induced sarcomas in the rat and the Chinese hamster, these findings strongly indicated that each oncogenic agent induces a specific karyotypic pattern (*Mitelman et al* 1972) Thus, the similar, non random karyotypic findings in different polyps of a single patient (the present case) and in single polyps from different patients (the cases cited) might be an indication of similar carcinogen(s) Further studies are necessary to throw light on this intriguing question, and to elucidate whether there is also a preferred karyotypic evolution in tumours derived from the same tissue (*Mark* 1971)

This work has been supported by grants from the Swedish Cancer Society, the John and Augusta Persson Foundation for Medical Research and by grants from the University of Lund, Sweden

REFERENCES

1 *Atkin N B* Cytogenetic studies on human tumors and premalignant lesions The emergence of aneuploid cell lines and their relationship to the process of malignant transformation in man In Genetic concepts and neoplasia Williams and Wilkins, Baltimore, p 36–56 1970

2 *Baker, M C* & *Atkin, N B* Chromosome abnormalities in polyps and carcinomas of the large bowel Proc. Royal Soc Med Suppl to *63* 9–10, 1970

3 *Cripps, W H* Two cases of disseminated polypus of rectum Tr Path Soc London *33* 165–169, 1882

4 *Alm, T, Dencker, H Lunderquist, A, Mark, J, Mitelman, F, Norryd, C* & *Tranberg K G* Gardner s syndrom Acta Chir Scand, in press

5 *Enterline, H T* & *Arvan, D A* Chromosome constitution of adenoma and adenocarcinoma of the colon Cancer *20* 1746–1759, 1967

6 *Gardner, E J* & *Richards, R C* Multiple cutaneous and subcutaneous lesions occurring simultaneously with hereditary polyposis and osteomatosis Am J Human Genet 5 139-147 1953

7 *Granberg I* Chromosomes in preinvasive, microinvasive and invasive cervical carcinoma. Hereditas *68* 165–218, 1971

8 *Lubs, H A* & *Kotler, S* The prognostic significance of chromosome abnormalities in colon tumors Ann Internat Med 67 328-336 1967

9 *McKusick, V A* Genetic factors in intestinal polyposis J Am Med Ass *182* 271–277, 1962

10 *Mark, J* Chromosomal analysis of a human retinoblastoma. Acta Opthalm *48* 124–135, 1969

11 *Mark, J* Chromosomal characteristics of neurogenic tumours in adults Hereditas *68* 61–100, 1971

12 *Messinetti, S, Zelli, G P, Marcellino, L R* & *Alcini E* Benign and malignant epithelial tumors of the gastroenteric tract Chromosome analysis in study and diagnosis Cancer *21* 1000–1010, 1968

13 *Miles, C P* Chromosome analysis of solid tumors II Twenty six epithelial tumors Cancer *20* 1274–1287, 1967

14 *Mitelman, F, Mark, J, Levan, G* & *Levan A* Tumor etiology and chromosome pattern Science *146* 1340–1341, 1972

15 *Sandberg, A A* & *Hossfeld, D K* Chromosomal abnormalities in human neoplasia Ann Rev Med *21* 379–408 1970

16 *Yamada, K, Takagi, N* & *Sandberg, A A* Chromosomes and causation of human cancer and leukemia II Karyotypes of human solid tumors Cnacer *19* 1879–1890, 1966

Acta path. microbiol scand Section A 81, 91–94, 1973

TRANSPLANTABLE BREAST TUMOURS IN RATS

Studies on Hormone Response

STEN SANDER, ARNT JAKOBSEN and JORUNN SANDER

The Department of Surgical Research, Rikshospitalet, Oslo, Norway
Head Professor, Dr med Egil Amundsen

Breast cancers have been induced in female Fisher rats. This strain of animals was less susceptible to a development of tumours than the Sprague Dawley and the Wistar rats previously studied The induced breast cancers were originally hormone-responsive and could be transplanted to animals with intact ovarian function Transplantation of hormone-responsive tumours may result in the development of hormone-unresponsive transplant tumours, whereas the opposite was not observed It is argued that this change in hormone-response can be explained as a selection phenomenon rather than a transformation of the tumour during the transplant procedure The observed change in hormone response was paralleled by a change in tumour morphology

Breast tumours induced in rats by administration of carcinogenic hydrocarbons have been widely used as an experimental model (2, 4, 5, 7, 9) The induction of tumours in female rats is not possible in the absence of oestrogenic hormones When established, the majority of these tumours are hormone-responsive, further growth depends on stimulation by endogenous or exogenous oestrogenic hormones. A minor group of such tumours, however, is essentially independent of oestrogenes and, if first established, will continue to grow after the host has been spayed The hormone-unresponsive tumours may represent separate tumour populations immediately after induction, or, hormone responsiveness may be a characteristic gradually lost by some breast tumours Changes in hormone-response can be studied by transplantation of tumours in an inbred strain of rats

Received 2 ix.72 Accepted 9 ix.72

MATERIAL AND METHODS

Strain Female inbred rats (Fisher—S P D animals) were supplied by Møllegaards Avlslaboratorium A/S, Ejby, Denmark, and used when they were 2–3 months old

Induction of breast tumours At the age of 55 days, the experimental animals were given 3 mg of 7,12 dimethylbenz (a) anthracene (DMBA) in lipid emulsion by the intravenous route (4, 7) The injection was repeated 3 days later

The animals were housed four in each cage and fed our routine diet they were examined twice a week for tumours Any palpable tumour was plotted and the growth of the tumours was assessed by measuring two diameters with calipers through the skin

Transplantation Surgery was performed in a sterile environment on rats anaesthetized by ether Tumours for transplantation were kept in sterile Krebs Ringer phosphate buffer, and necrotic and fibrous tissue dissected away Four tiny pieces of tumour tissue (2 × 2 mm) were transplanted to the subcutaneous tissue on the back of each recipient. Tunnels in four directions for the introduction of the tumour were made through a central incision in a shaved area of the skin Silk thread was used for closure of the incisions

Ovariectomy In order to test the hormone re sponse the animals were ovariectomized *when the* tumours exceeded 8 mm in diameter The tumours were then measured every third day and subse quently classified as hormone responsive or hor mone unresponsive according to continued growth or regression as previously described (7) All tu mours and grossly abnormal tissue were fixed in 4 per cent glutaraldehyde and prepared *for histo*logical examination Sections were cut at 5 μ from paraffin blocks and stained with haematoxylin and eosin

Sources of error We have experienced failures with the transplantations, in some series none of the animals developed tumours This may be due to necrosis or infection of the transplant tissue Vital dye tests of tumour suspensions have shown that a considerable number of tumour cells are destroyed during the transplantation procedure Careful selection and handling of donor tissue is essential

RESULTS

Experiment 1 Among the 13 animals which received DMBA, only 3 developed tumours suitable for transplantation after a latency period of 2–3 months These tumours were hormoneresponsive

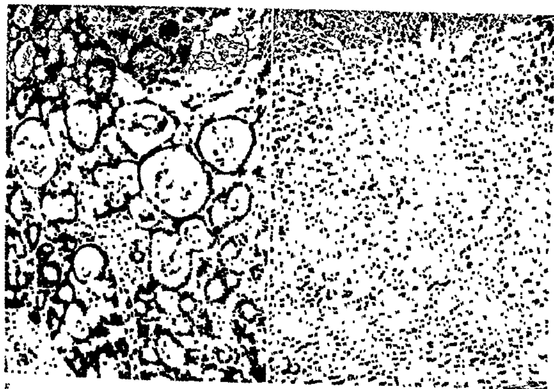
Experiment 2 One tumour induced in experiment 1 was transplanted to 7 ovariec tomized recipients and 3 controls None of the ovariectomized recipients developed tu mour, whereas tumours developed in the 3 controls

Experiment 3 One *hormone responsive tu mour,* in a state of regression after ovariec tomy of the host, was transplanted to 5 reci pients The 5 recipient animals developed 12 tumours As soon a the tumours had achieved the appropriate size, ovariectomy was carried out Following ovariectomy of the recipient animals it became apparent that all the trans plant tumours were *hormoneunresponsive* These tumours were followed for several weeks, none of these metastasized but gra dually, necrosis haemorrhage and ulceration of the skin was observed Further trans plantation of these tumours was difficult In two series including eight animals none of the recipients developed tumours In a third series, donor tissue was carefully selected from the subcapsular area of a tumour and transplanted to three animals all of which developed tumours These three transplant tumours were *hormone unresponsive*

Experiment 4 One of the transplant tu mours in experiment 2 was *transplanted to 4 recipients* at the same time as the donor animal was ovariectomized Half of the tu mour was taken for transplantation while the rest of the tumour was left intact for deter mination of its hormone response Great care was taken to avoid interference with the blood supply of this latter part of the tumour In this case we used donor tissue that was not in a state of atrophy The donor tumour was later classified as hormone responsive Transplant tumours developed in three out of the four recipient animals, these tumours were all hormone responsive like the donor tumour utilized in this case

Histology

The histological characteristics of breast tumours induced by DMBA have been ab undantly described in the literature (10) The tumours induced in our Fisher rats were predominantly adenocarcinomas with various grades of differentiation Most common were tumours dominated by acinar forms, tubular growth or more uniform sheets of anaplastic cells Some tumours were mixtures of differ ent morphological types Fibro adenomas were not found in this series The stroma in the tumours varied considerably from thin fibrous septa to thick fibrous bands In the stroma between the lobules and in the peri phery of the tumours infiltration of lympho cytes was frequently found A varying extent of necrosis was observed In the hormone re sponsive tumours flattening of the epithe lium with increase in the size of acinar lumen was a common finding during the process of regression Particular attention was paid to the tumours in experiment 3 (Fig 1a and b) The donor tumour was an adenocarcinoma with luminated glandular structure The transplant tumours however were less dif ferentiated with only remnants of glandular structure These tumours were dominated by



F . . ui in experiment 3 X 440

uni ...s of more anaplastic cells divided by thin fibrous strands of stroma Necrosis and chronic inflammatory cells were present In experiment 4, donor tumour and transplant tumours were essentially adenocarcinomas of similar morphology

DISCUSSION

Following administration of DMBA, rats are very susceptible to the development of breast cancer There are, however, apparent differences between the strains commonly used in laboratories (2) We used Fisher rats in our experiments because an inbred well controlled strain of S P D animals was at our disposal. According to our own experience with the Sprague-Dawley and the Wistar strains, it appeared that the Fisher rat was less susceptible to the carcinogenic effect of DMBA In experiment 1 only three animals out of thirteen developed breast cancer whereas the incidence of tumours in Sprague-Dawley is more than 90 per cent if these high doses of DMBA are given

Our results in experiment 2 confirm that the tumours originally induced by DMBA were hormone-responsive in character This is in agreement with the concept that hormone-responsive tissues transplanted to castrated animals will fail to grow The tumours transplanted in this experiment were dependent upon intact ovarian function in the recipient animals These tumours were used for further experiments

The DMBA induced breast cancers are essentially adenocarcinomas but are not always homogenous in structure Frequently, there are areas of different morphological types within a single tumour which show various grades of differentiation If such tumour shrinks after ovariectomy it is possible that it is mainly the hormone-responsive cells that turn atrophic whereas hormone unresponsive

Ovariectomy In order to test the hormone response the animals were ovariectomized when the tumours exceeded 8 mm in diameter The tumours were then measured every third day and subsequently classified as hormone responsive or hormone unresponsive according to continued growth or regression as previously described (7) All tumours and grossly abnormal tissue were fixed in 4 per cent glutaraldehyde and prepared for histological examination Sections were cut at 5 μ from paraffin blocks and stained with haematoxylin and eosin

Sources of error We have experienced failures with the transplantations, in some series none of the animals developed tumours This may be due to necrosis or infection of the transplant tissue Vital dye tests of tumour suspensions have shown that a considerable number of tumour cells are destroyed during the transplantation procedure Careful selection and handling of donor tissue is essential

RESULTS

Experiment 1 Among the 13 animals which received DMBA only 3 developed tumours suitable for transplantation after a latency period of 2 3 months These tumours were hormoneresponsive

Experiment 2 One tumour induced in experiment 1 was transplanted to 7 ovariectomized recipients and 3 controls None of the ovariectomized recipients developed tumour, whereas tumours developed in the 3 controls

Experiment 3 One *hormone responsive tumour*, in a state of regression after ovariectomy of the host, was transplanted to 5 recipients The 5 recipient animals developed 12 tumours As soon a the tumours had achieved the appropriate size ovariectomy was carried out Following ovariectomy of the recipient animals it became apparent that all the transplant tumours were *hormoneunresponsive* These tumours were followed for several weeks, none of these metastasized but gradually, necrosis haemorrhage and ulceration of the skin was observed Further transplantation of these tumours was difficult In two series including eight animals none of the recipients developed tumours In a third series, donor tissue was carefully selected from the subcapsular area of a tumour and transplanted to three animals, all of developed tumours These three tra tumours were *hormone unresponsive*

Experiment 4 One of the transpla mours in experiment 2 was transplanted recipients at the same time as the d animal was ovariectomized Half of tl mour was taken for transplantation wl il rest of the tumour was left intact for d mination of its hormone response Great c was taken to avoid interference with blood supply of this latter part of the tum In this case we used donor tissue that v not in a state of atrophy The donor tum was later classified as hormone respon s Transplant tumours developed in three o of the four recipient animals, these tumour were all hormone responsive like the dono tumour utilized in this case

Histology

The histological characteristics of breast tumours induced by DMBA have been ab undantly described in the literature (10) The tumours induced in our Fisher rats were predominantly adenocarcinomas with various grades of differentiation Most common were tumours dominated by acinar forms, tubular growth or more uniform sheets of anaplastic cells Some tumours were mixtures of different morphological types Fibro adenomas were not found in this series The stroma in the tumours varied considerably from thin fibrous septa to thick fibrous bands In the stroma between the lobules and in the periphery of the tumours infiltration of lymphocytes was frequently found A varying extent of necrosis was observed In the hormone responsive tumours flattening of the epithelium with increase in the size of acinar lumen was a common finding during the process of regression Particular attention was paid to the tumours in experiment 3 (Fig 1a and b) The donor tumour was an adenocarcinoma with luminated glandular structure The transplant tumours however were less differentiated with only remnants of glandular structure These tumours were dominated by

Acta path. microbiol. scand. Section A 81, 97–111, 1973

QUANTIFICATION OF HUMAN SEMINIFEROUS EPITHELIUM

III. Histological Studies in 44 Infertile Men with Normal Chromosome Complements

NIELS E. SKAKKEBÆK, RICH HAMMEN, JOHN PHILIP and HEINRICH REBBE

Chromosome Laboratory and Fertility Clinic, Department of Obstetrics and Gynaecology, Rigshospitalet, University of Copenhagen, Denmark

Fortyfour infertile 46,XY men with severe deficiencies of the spermiogram were investigated by quantitative analyses of the seminiferous epithelium employing the Sertoli cell number as a basis for reference. The following five groups of germ cells were recorded separately: 1) Spermatogonia, 2) preleptotene + leptotene spermatocytes, 3) zygotene + pachytene spermatocytes, 4) Sa + Sb spermatids and 5) Sc + Sd spermatids. The results were compared with previous results from similar studies in controls. Four of the patients had normal or only slightly impaired spermatogenesis, while the rest showed substantial abnormalities. In 21 cases an isolated reduction in the number of late or both early and late spermatids was found. In six other patients also the number of spermatogonia and/or spermatocytes was reduced. The remaining 13 cases showed heterogeneous pictures composed of Sertoli cell only tubules, tubules with germ cells and partly or totally obliterated tubules. Males with different types of testicular lesions often showed the same degree of oligospermia. Although several of the patients showed abnormal features that may interfere with fertility, it was not possible to correlate failure of the testicle to descend, varicocele, abnormal hair distribution or abnormal excretion of 17-ketosteroid fractions to any characteristic trait in the seminiferous epithelium. The urinary excretion of total gonadotrophins was within normal limits in all but two of the cases. Only six of the patients had small sized testes.

Although cytogenetic and endocrinological studies have improved our knowledge regarding the aetiology of testicular disorders in infertile men, the majority of infertile men have no detectable chromosomal or endocrinological disorders (*Paulsen* 1968). A large part of these men have, however, severe quantitative changes of the cells of the seminiferous epithelium in all or some of the tubules.

Investigation of such cases should reveal at what level(s) in spermatogenesis a possible disorder may be located. Due to the variability of the seminiferous epithelium even in men considered to have normal spermatogenesis, quantitative analysis of the different types of germ cells is important in revealing and classifying such defects (*Roosen-Runge* 1956, *Steinberger & Tjioe* 1968, *Paulsen* 1968, *Rowley & Heller* 1971, *Skakkebæk & Heller*, 1973 a).

Received 15.viii.72. Accepted 4.x.72.

Requests for reprints should be addressed to: N. E. Skakkebæk, Chromosome Laboratory, Rigshospitalet, Copenhagen Ø, Denmark.

The present communication reports data from quantitative studies on the cells of the seminiferous epithelium in a series of infertile men with normal chromosome complements The results are compared with results from similar studies in a control group (*Skakkebæk & Heller*, 1973 a)

MATERIALS

Forty four men aged 25–51 years (average 32 2 years) with normal chromosome complements were investigated They were found in a group of 93 patients referred to our fertility clinic for testicular biopsy because of aspermia, "severely impaired fertility" or "very severely impaired fertility" (*Hammen* 1944) At least three semen specimens were analysed, except in cases where semen analyses had been carried out elsewhere, then only one or two were investigated All patients had severe deficiences of one or more, in most cases all of the three main qualities of the semen (number, motility and morphology) Four cases (Case 1, 9, 15, and 25) had aspermia One patient (Case 31) had an average sperm count of 64 mill/ml, the remaining had counts ranging from 0 1 to 37 0 mill/ml in average 8 0 mill/ml

TABLE 1 *The Material*

	No of cases
Originally referred	93
Chromosome abnormalities	6
Sertoli cell only syndrome	5
Obliterated tubules	3
Abnormal cell morphology	1
Unsuitable biopsies	34
Total excluded	49
Present material	44

The original material was reduced in size for different reasons (Table 1) Results from studies in patients with chromosome disorders were reported separately (*Philip et al* 1970 *Skakkebæk* 1969 *Skakkebæk et al* 1970 *Skakkebæk et al* 1973b) Patients with the complete Sertoli cell only picture or with obliteration of all tubules and patients who showed a new type of abnormal cell morphology (*Skakkebæk* 1972) were excluded A number of biopsies were unsuitable, either because of small size or [illegible]ccessfull preparation The exclusion of [illegible] le biopsies was made on a blind basi[illegible] h course of the project the biopsy [illegible] ed considerably, as 27 of the unsuitable biopsies originated from the first half of the study

A medical history and a physical examination was obtained prior to biopsy in all cases Examination for varicocele, hernia hydrocele abnormal secondary sex characteristics unusual testicular size (15 cm³>size>35 cm³) and failure of testicular descent was included in the physical examination

METHODS

Testicular biopsies approximately 3–4 mm in diameter, were removed surgically under local anaesthesia Generally biopsies were taken from the right testis The biopsies were fixed in Stieve's fixative, embedded in paraffin sectioned serially at 4 μ and stained with iron hematoxylin according to the method of *Rowley & Heller* (1966)

The tubule method of *Rowley & Heller* (1971) was used for quantification of the germ cells The different types of spermatogonia were grouped together Spermatocytes and spermatids were classified according to the description of the seminiferous epithelium given by *Clermont* (1963) and *Heller & Clermont* (1964) All microscopies were performed by the first author

In biopsies with one category of tubules (homogenous picture) a differential count of germ cells and Sertoli cells was carried out on 30 randomly chosen cross sections of tubules (except in Case 5 where only 21 adequate tubules were found) The total number of counted germ cells of the different types were divided by the total number of Sertoli cells found in the same areas The values are referred to as the *Sertoli cell ratio (SCR)*

In biopsies with different categories of tubules (heterogeneous picture) an estimate of the distribution of tubules in the different categories (Sertoli cell only tubules tubules with preserved spermatogenesis etc) was determined by classification of all tubules in a randomly chosen section of a biopsy The relative frequency of the different tubule types was then expressed as percentage of all tubules in this section (Table 3)

Separate counts were made on 30 randomly chosen sections from that category of tubules in which all types of germ cells were present (except in Case 32 where the count was performed on tubules with an almost total arrest of spermatogenesis at spermatocyte level)

Estimates of testicular size were obtained by use of an orchidometer (a calibrated series of models of testes) Urinary excretion of gonadotrophins and 17 ket[illegible] fractions were measured routinely pr[illegible] [illegible]psy (*Johnsen* 1956 a 1956 b 1958 1959[illegible]) Normal [illegible] the excretion of gonadotrophins is [illegible] MUU/24 h in average



Fig 1 a Section showing spermatogenesis in control individual × 75

b Section showing quantitatively normal spermatogenesis in Case 25 Note that some of the spermatids are morphologically abnormal × 875

men and 3–40 in ideal men (*Johnsen* 1971) Normal ranges for the excretion of fractionated 17 ketosteroids are the following for men aged 20–40 years DHA fraction 1.27–8.28 mg/24 h androsterone (A) + etiocholanolone (E) fraction 4.77–15.67 mg/24 h and the A/E ratio 0.45–2.13 (*Johnsen et al* 1971) Other fractions were not considered in this study

RESULTS

All results obtained were compared with the Sertoli cell ratio obtained from a study of 21 men with normal sperm counts (> 60 mill/ml) and without a history of reproductive failure (*Skakkebæk* & *Heller*, 1973 a) (Fig 1 a Table 2) Accordingly the patients could be classified into different groups as seen below

Biopsies with One Category of Tubules

Group 1 Three cases (4 16 24) showed a *distinct reduction in number of spermatocytes and spermatids and a slight reduction in the number of spermatogonia* (Fig 2, Fig 3 a b Table 2)

Group 2 In three cases (15 19, 29) *a reduction in number of spermatocytes and spermatids* was found (Fig 4, Fig 3 c, d, Table 2)

Group 3 Eight cases (2 5 6 7, 9, 18, 23, 26) showed a *reduced number of early and*

TABLE 2 *SCR and Total Sertoli Cell Number Obtained from Counts on Biopsies with a homogeneous picture*§

Case	Spermatogonia	Spermatocytes PL	Spermatocytes L	Spermatocytes Z+P	Spermatids Sa+Sb	Spermatids Sc+Sd	S
1	1 41	0 18	0 25	2 14	2 88	1 39	352
2	1 60	0 16	0 15	1 71	1 51	0 69	357
3	2 33	0 31	0 46	3 04	3 95	3 25	277
4	0 99	0 11	0 07	0 49	0 11	0 04	491
5	1 79	0 29	0 21	1 60	0 07	0 03	275
6	1 68	0 24	0 17	1 70	1 24	0 83	427
7	1 73	0 32	0 23	2 15	1 56	0 46	342
8	1 54	0 22	0 19	1 75	1 90	0 65	303
9	2 24	0 22	0 16	2 67	0 07	0	323
10	1 35	0 20	0 13	1 36	2 02	0 74	442
11	2 00	0 21	0 13	1 01	2 03	0 89	313
12	1 68	0 25	0 14	1 45	2 08	1 59	380
13	1 85	0 36	0 21	2 00	3 88	2 16	275
14	*1 30*	*0 11*	*0 18*	*1 54*	*1 85*	*0 98*	412
15	1 02	0 16	0 05	0 64	0 18	0 13	248
16	0 96	0 11	0 04	0 72	0 70	0 30	407
17	1 92	0 21	0 13	2 46	2 48	0 46	320
18	1 62	0 19	0 16	1 72	1 28	0 34	394
19	1 59	0 13	0 17	0 87	0 39	0 12	369
20	1 31	0 17	0 19	1 13	1 77	1 12	366
21	2 05	0 16	0 10	1 68	2 65	1 03	288
22	1 47	0 08	0 11	1 75	1 57	0 85	334
23	1 17	0 44	0 18	1 12	0 10	0 01	561
24	0 94	0 07	0 06	0 63	0 74	0 31	509
25	1 45	0 21	0 15	2 27	2 61	1 43	344
26	1 31	0 14	0 30	1 69	0 27	0 15	287
27	1 30	0 14	0 23	1 61	2 16	0 98	328
28	1 92	0 37	0 39	2 99	2 04	1 33	262
29	1 35	0 04	0 16	0 67	1 25	0 68	472
30	1 54	0 20	0 22	2 05	2 84	1 23	378
31	1 57	0 16	0 17	1 48	1 95	1 07	412
Control group mean	1 77	0 25	0 22	1 96	3 05	2 14	
Control group range	1 05 – 2 83	0 06 – 0 44	0 08 – 0 38	1 03 – 2 86	1 59 – 4 87	1 44 – 3 63	

§ PL-preleptotene, L leptotene, Z-zygotene and P pachytene spermatocytes Sa, Sb Sc and Sd are consecutive steps in the maturation process of spermatids S – Sertoli cells

late spermatids (Fig 5, Fig 6 a, b, Table 2) A relatively high number of preleptotene spermatocytes were found in Case 23 The preleptotene/spermatogonia ratio was in this case 0 37 (normal range 0 6–0 24, *Skakkebæk* & *Heller*, 1973 a)

Group 4 A quantitatively normal spermatogenesis until early spermatid level but with *reduced number of late spermatids* was found in 13 cases (8, 10 11, 12, 14, 17, 20, 21, 22, 27, 28, 30, 31) (Fig 7, Fig 6 c, d, Table 2) Although the number of early spermatids for patients of this group fell within the normal range, the group as a whole showed a reduced number of this type of cells in comparison with the controls The Mann-Whitney test showed a significant difference at the P = 0 05 level

Group 5 Four cases (1, 3, 13 25) showed *normal or near normal spermatogenesis* (Fig 8, Fig 1 b, Table 2) Two of these (1 and 25) possibly had an obstruction of the egress

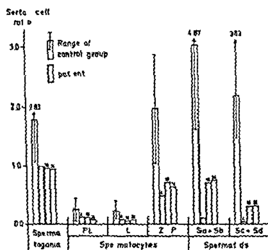


Fig 2 SCR of different groups of germ cells in cases with a distinct reduction in number of spermatocytes and spermat ds and presumed in hibition at spermatogonial level Comparison with a control group of fertile 46 XY men (*Skakkebæk & Heller* 1973 a) The range of the control va ues are equivalent to estimates of the 2 5 and 97 5 percentiles of the frequency distribut on for the fertile men The numbers on the top of the columns ind cate the case numbers

of sperm in the ductal system Both had aspermia and one had a history of infection of the epididymis and no vasa deferentia were palpable in the other subject The other two cases (3 and 13) with sperm counts of 16 5 and 27 5 mill/ml each had, however, only one normal sized testis (the biopsy originated from this side) and a small testis located in the top of scrotum

Biopsies with More than One Category of Tubules

Spermatogenesis in cases with partial 'Ser toli cell-only' syndrome as seen from Table 3 six biopsies contained Sertoli cell-only' tubules in addition to tubules with spermato genesis They were found mixed with tubules with so called spermatogenic arrest in Case 32 (Fig 9 a b), in Case 36 with tubules con taining a reduced number of both spermatocytes and spermatids, in Case 34, 35, and 38 with tubules containing a reduced number of late spermatids and in Case 39 with apparent ly normal tubules

Spermatogenesis in cases with a substantial number of obliterated tubules in three of

TABLE 3 *Percentage§ of Different Categories of Tubules in Cases where the Testicular Biopsy Showed a Heterogeneous Picture*

Case	Sertoli cells only	Sertoli cells and spermatogonia	Sertoli cells spermatogonia and spermatocytes	All types of germ cells*	Totally or partly obliterated tubules
32	38		62*		
33			8	86	6
34	13	5		76	6
35	79			21	
36	48			52	
37				90	10
38	36			50	14
39	74			15	11
40				88	12
41		15		57	28
42				84	16
43				95	5
44				37	63

* A d fferent al count was performed on germ cells in tubules of this type (Table 4)
§ These percentages are based on counts of between 81 and 342 tubules in a randomly chosen section of a b opsy

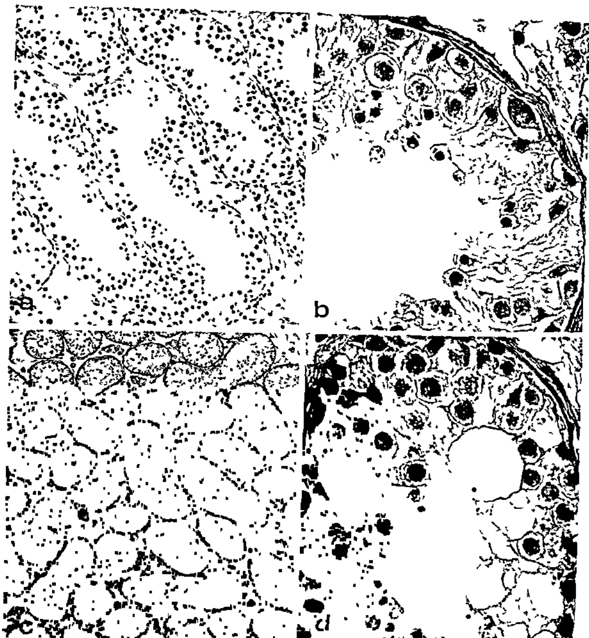


Fig 3 a + b Section showing spermatogenesis in Case 16 Note reduction in number of all types of germ cells a × 115, b × 525
c + d Section showing spermatogenesis in Case 19 Note the reduction in number of spermatocytes and spermatids c × 60 d × 525

the cases mentioned above with partial "Sertoli cell-only" syndrome obliterated tubules were also seen (Case 34, 38 and 39, Table 3) Such lesions were found to be the principal defect in Cases 33, 37, 40, 41 42, 43, and 44 (Fig 9 c) The remaining tubules in the latter cases showed the following characteristics in Case 43 the SCR of all types of germ cells were reduced Case 37 contained a reduced number of spermatocytes and spermatids A decreased SCR of early and late spermatids was found in Cases 40 and 41 A slight decrease in SCR of late spermatids was found in Case 44 while quantitatively normal

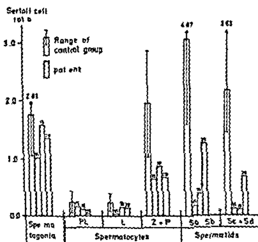


Fig 4 SCR of different groups of germ cells in cases with a moderate reduction in number of spermatocytes and a severe reduction in numbers of spermatids in comparison with the control group The numbers on the top of the columns indicate the case numbers

spermatogenesis apparently was present in Cases 33 and 42 (Table 4)

The Accuracy of the Method for Quantification of the Seminiferous Epithelium

In order to evaluate the accuracy of the method for quantification of the seminiferous epithelium in studies of patients with testicular disorders, the standard error of a mean (SEM) for Sertoli cell ratios of the various types of germ cells within the different groups of patients were calculated and compared with corresponding figures from counts in controls The calculation procedure was the same in the previous study (*Skakkebæk & Heller*, 1973 a).

As seen from Table 5 the SEM for the Sertoli cell ratios of the various types of germ cells of counts in patients were either equal to or lower than the respective figures in the control group The low values were found in the group of patients with a low frequency of the respective cell types Thus, it can be concluded that the Sertoli cell ratios found in patients were determined at least as accurately as in the group of fertile men

The Relationship between the Results of the Quantitative Analysis of the Seminiferous Epithelium and Clinical Traits

Failure of testicular descent was found among cases with a wide range of abnormalities Of these, Cases 10, 11, and 14 were demonstrated to have reduced numbers of spermatids Case 29 had a reduced number of both spermatocytes and spermatids and Cases 36 37 and 39 showed a heterogeneous

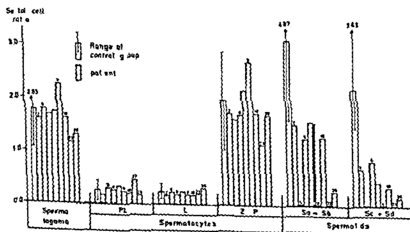


Fig 5 SCR of different groups of germ cells in cases with a reduction in number of early and late spermatids compared with the control group The numbers on the top of the columns indicate the case numbers.

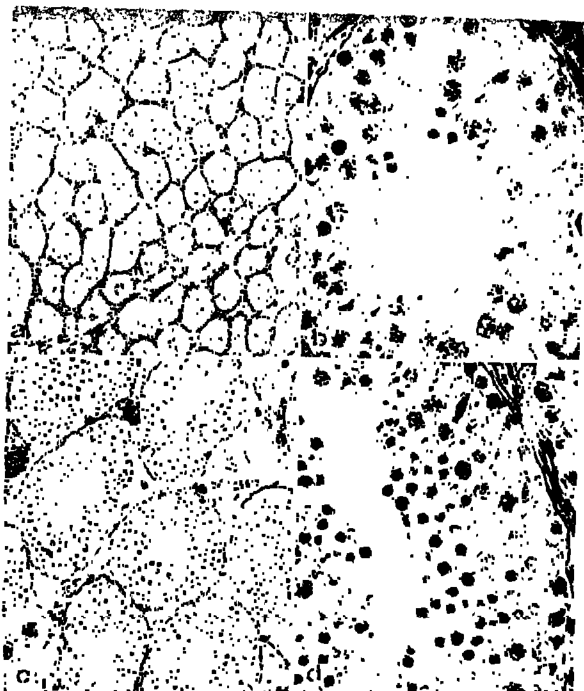


Fig 6 a + b Section showing spermatogenesis in Case 26 Note the reduction in number of early and late spermatids a × 60, b × 525
c + d Section showing spermatogenesis in Case 30 Note the numerous germ cells of all types except late spermatids c × 115, d × 525

picture with obliterated tubules and tubules with complete spermatogenesis

Some of the cases had a history of *varicocele* or a varicocele was present Of these, Case 20, 21 and 30 showed a reduced number of late spermatids, Case 5 a reduced number of both early and late spermatids, while Case 4 showed a reduction in the number of all

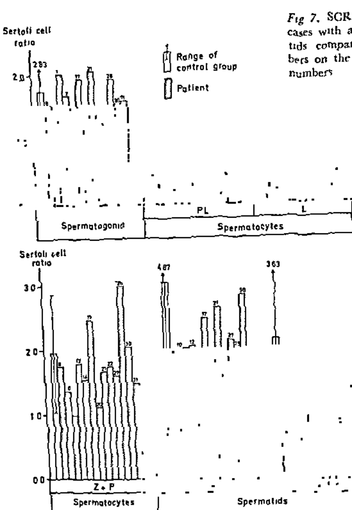


Fig 7. SCR of different groups of germ cells in cases with a reduction in number of late spermatids compared with the control group The numbers on the top of the columns indicate the case numbers

TABLE 4 *Results of Quantitative Analyses on a Selected Tubule Category in Cases with a Heterogenous Picture, Sertoli Cell Ratio and Sertoli Cell Number (cf Table 3)*

Case	Spermatogonia	Spermatocytes PL	Spermatocytes L	Spermatocytes Z + P	Spermatids Sa + Sb	Spermatids Sc + Sd	S
32	1 12	0 07	0 12	0 76	0 08	0 11	501
33	1 52	0 19	0 12	1 64	2 22	1 71	371
34	2 46	0 35	0 23	1 95	2 76	0 62	231
35	1 51	0 20	0 15	1 59	2 08	1 24	366
36	1 25	0 10	0 18	0 91	0 78	0 36	476
37	1 40	0 26	0 38	0 95	0 41	0 16	363
38	1 16	0 17	0 09	1 31	1 83	1 21	281
39	1 76	0 26	0 13	1 34	2 77	1 56	441
40	1 63	0 20	0 15	1 82	1 55	0 73	268
41	1 86	0 19	0 32	1 99	1 49	0 29	370
42	2 82	0 25	0 23	2 37	2 68	1 45	368
43	0 71	0 02	0 04	0 39	0 42	0 35	501
44	2 43	0 34	0 32	2 62	1 60	1 36	247

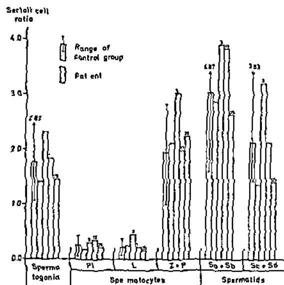


Fig 8 SCR of the different *groups of germ cells* in cases with normal or slightly impaired spermatogenesis, compared with the control group The numbers on the top of the columns indicate the case numbers

types of germ cells A small varicocele was present in Case 13, who had quantitatively normal or near normal spermatogenesis

Abnormal hair distribution was found in cases with different types of abnormalities of the seminiferous epithelium This symptom was present in two cases with a reduced number of late spermatids (27 and 28), three cases with a reduced *number of early* and late spermatids (5, 18, 23), two cases with a reduced number of spermatocytes and spermatids (15 and 29), and one case with a presumed reduction in number of all cell types (Case 4)

The *urinary excretion of "total gonadotrophins"* was significantly increased (90 MUU/24 h) in one of the patients with a low number of late spermatids (Case 28 *)) Another patient (Case 6), who showed a reduced number of both early and late spermatids, had a relatively low excretion of gonadotrophins (3 and 4 MUU/24 h *on two* occasions) The rest of the patients showed values within normal ranges (5 75 MUU/24 h) although two patients (Case 40 and 42) had values above the range of 'ideal' men (3–40 MUU/24 h) Average excretion of total gonadotrophins was 24 MUU/24 h which corresponds well with the average *excretion of* 20 MUU/24 h *in normal* controls

*) *One analysis only was performed*

TABLE 5 *The SEM for Sertoli Cell Ratios of the Various Types of Germ Cells Estimated for the Different Groups of Individuals*

Group of individuals	Spermatogonia	Spermatocytes *PL + L*	*Z + P*	Spermatids *Sa + Sb*	*Sc Sd*
Control group of 21 men (Skakkebæk & Heller 1973 a)	0 13	0 10	0 19	0 35	0 26
13 patients with reduced number of late spermatids	0 14	0 08	0 16	0 29	0 15
8 patients with reduced number of both early and late spermatids	0 10	0 08	0 16	(0 12)*	(0 11)*
3 patients with reduced number of spermatocytes and spermatids	0 07	0 02	0 14	(0 18)*	(0 08)*
4 patients with normal or near normal number of all types of germ cells	0 18	0 10	0 10	0 46	0 29
13 patients with different categories of tubules§	0 10	0 09	0 16	0 27	0 21

* These estimates are of limited value because some of the corresponding Sertoli cell ratios were extremely low

§ For this group the SEM is calculated from counts from the category of tubules with most advanced spermatogenesis

DISCUSSION

Various Pathological Pictures of the Seminiferous Epithelium

The variation in the pattern of the seminiferous epithelium within and between the individuals in the present study was considerable

Some variation in the pattern of the germinal epithelium is always present, even in fertile men considered to have normal spermatogenesis A cycle of the seminiferous epithelium has been described in the human testis (*Clermont 1963*) This may account for some of the variations in stage III of the cycle, for instance, the spermatids of type Sb^1 are the most advanced germ cells, while in stage II mature spermatozoa are present Some variation in tubular diameter is normally found, also, and the total number of germ cells in a cross section of a tubule may, therefore, vary considerably Furthermore, the human seminiferous epithelium is so vulnerable that even when biopsies are handled with extreme care, the epithelium may be somewhat damaged The terms "homogeneous picture' and 'heterogeneous picture" may, therefore, need some comments The term "homogeneous picture" in our

cells in 40 of the 44 patients (91 per cent) deviated from the control group, whereas four cases did not deviate significantly In the majority of the patients only one category of tubules was found ('homogeneous picture') The most common finding in this group was a reduction in the number of spermatids, which was present in 21 cases In 13 of these patients the SCR of late spermatids fell below the range of the controls, while SCR of early spermatids was within normal range A comparison between this group of 13 patients and the control group revealed, however, that early spermatid level must have been the site of impairment in at least some of these patients also In other cases, inhibition of spermatogenesis occurred at earlier levels, *i e* at spermatocyte or spermatogonia level It is noteworthy that some of these cases showed a substantial number of spermatids

Thirteen of the patients showed heterogeneous pictures with two or more different categories of tubules, *e g* combination of Sertoli cell only tubules and tubules with spermatogenesis In these biopsies the tubules with spermatogenesis preserved showed variable pictures In one case (32) almost no spermatids were present while the other cases

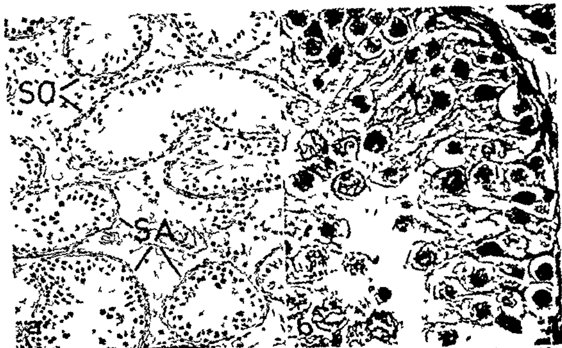


Fig 9 a + b Sect ons of test cular b ops es from Case 32 a Note the heterogeneous p cture com po ed by Sertoli cell only tubules (SO) and tubules with an arrest at spermatocyte level (SA) × 125 b H gh power magnif cat on of a tubule w th arrest at primary spermatocyte level × 575

c Section of test cular b opsy from Case 44 Note the heterogeneous p cture composed by part ly or totally obl tera ed tubules (ind cated by ar rows) and tubules w th preserved spermatogeneses × 75

The DHA (dehydro soandrosterone) ex cretion was normal in all cases except in Ca e 39 with s gnificantly increased excre tion (9 70 mg/24 h) and in Case 3*) 36*) and 41 with significantly decreased values The DHA values in these three cases were 1 08 1 18 and 0 95 respectively (normal l mits 1 27 8 28 mg/24 h) The values for androsterone + ethiocholanolone (A + E) fraction of the 17 KS and the A/E ratio were w thin the normal range in all cases except Case 30*) and 31*) which showed s gni ficantly increased A/E ratios (4 00 and 2 59 mg/24 h respectively normal range 0 45–2 13 mg/24 h)

* One analys s o ly was performed

An unusual *si e of testes* was found in seven patients only Five cases (5 13 20 29 and 36) had one small testis measuring 12 8 8 12 and 8 cm³ respectively Except in Case 20 the biopsy originated from the normally sized testis In four cases the testes were examined without the use of an orchidometer and all were estimated to have normal sized testes except Case 3 who had both a small and a normally sized gonad One patient (Case 31) had unusually large testes both measuring 40 cm³ In the remaining cases no gonad was larger than 30 cm³ The aver age testicular s ze was 19 7 cm³ In two cases (11 and 14) only one testis was palpable at the physical examination

DISCUSSION

Various Pathological Pictures of the Seminiferous Epithelium

The variation in the pattern of the seminiferous epithelium within and between the individuals in the present study was considerable

Some variation in the pattern of the germinal epithelium is always present, even in fertile men considered to have normal spermatogenesis A cycle of the seminiferous epithelium has been described in the human testis (*Clermont* 1963) This may account for some of the variations in stage III of the cycle, for instance, the spermatids of type Sb[1] are the most advanced germ cells, while in stage II mature spermatozoa are present Some variation in tubular diameter is normally found, also, and the total number of germ cells in a cross section of a tubule may, therefore, vary considerably Furthermore, the human seminiferous epithelium is so vulnerable that even when biopsies are handled with extreme care, the epithelium may be somewhat damaged The terms "homogeneous picture" and "heterogeneous picture" may, therefore, need some comments The term "homogeneous picture" in our study refers to biopsies with a variation of the seminiferous epithelium which the investigator could not distinguish from the variation in control biopsies The term "heterogeneous picture", on the other hand, refers to biopsies in which distinctly different categories of tubules were present, as for instance obliterated tubules and tubules with preserved spermatogenesis

In biopsies with a heterogeneous picture tubules of one category very often were located in groups (Fig 9 a) Since the volume of a testicular biopsy is relatively small the biopsy may not always be representative for the whole testis *Tillinger* (1957), however, studied the problem and concluded that the testicular biopsy with very few exceptions reflects the condition of the whole organ

The SCR of the different groups of germ cells in 40 of the 44 patients (91 per cent) deviated from the control group, whereas four cases did not deviate significantly In the majority of the patients only one category of tubules was found ("homogeneous picture") The most common finding in this group was a reduction in the number of spermatids, which was present in 21 cases In 13 of these patients the SCR of late spermatids fell below the range of the controls, while SCR of early spermatids was within normal range A comparison between this group of 13 patients and the control group revealed, however, that early spermatid level must have been the site of impairment in at least some of these patients also In other cases, inhibition of spermatogenesis occurred at earlier levels, i e at spermatocyte or spermatogonia level It is noteworthy that some of these cases showed a substantial number of spermatids

Thirteen of the patients showed "heterogeneous pictures" with two or more different categories of tubules, e g combination of Sertoli cell-only tubules and tubules with spermatogenesis In these biopsies the tubules with spermatogenesis preserved showed variable pictures In one case (32) almost no spermatids were present while the other cases showed a varying number of all types of germ cells and three of these patients (33 39, and 42) demonstrated quantitatively normal spermatogenesis in a significant proportion of the tubules

Relationship between Analysis of the Seminiferous Epithelium and of the Seminal Fluid

In spite of the above mentioned biases, the testicular biopsy is important in the search for the causes of aspermia and oligospermia because deficiencies of the spermiogram may be due to total or partial obstruction of the egress of sperm as well as to testicular failure Furthermore it is not possible to predict the nature of a testicular impairment from the results of sperm analyses since males with the same degree of oligospermia often show quite

different composition of the seminiferous epithelium, *e g* Cases 19 28 and 38 all showed severe oligospermia (0 4 2 2 mill/ml), although Case 19 contained a reduced number of both spermatocytes and spermatids, Case 28 showed an impairment at late spermatid level and Case 38 showed a heterogeneous picture composed by obliterated tubules, Sertoli cell-only tubules and tubules with preserved spermatogenesis

A lack of correlation between results of the sperm analyses and quantitative analysis of germ cells was found by *Roosen Runge et al* (1957) in an investigation of infertile men Recently *Heller & Heller* (1970) reported a similar finding in a study of normal men who were treated with clomiphene High doses were able to cause a decrease in sperm count down to zero in spite of the presence of many mature spermatids in the testes These authors suggested that degenerating germ cells may have been absorbed by the Sertoli cells or the epididymis and as a consequence no sperm are found in the ejaculate

It would of course be valuable to record morphologically abnormal spermatids in a quantitative manner since abnormal cells may be more likely to undergo degeneration before they appear in the ejaculate However distinction between normal and abnormal forms is difficult in histological sections and registration of abnormal forms was therefore omitted in the present study although their frequency might however, be considerably increased in some of the patients

Relationship between Analysis of the Seminiferous Epithelium and Other Examinations of the Patients

The patients of the present investigation were referred for testicular biopsy because of severe failure of the semen cytology Several of the patients had a history of disorders or the examination revealed abnormalities that may interfere with fertility

Nine of the cases had a history of failure of testicular descent Two of these showed a partial Sertoli cell only picture as earlier described by *Johnsen* (1970) No characteristic feature of the testicular biopsy was found in the remaining cases Varicocele was found in six other men showing a wide range of abnormalities The varicocele may be causally related to testicular failure in all of these cases although a relatively large proportion of men with varicocele may have high sperm counts (*Hotchiss* 1970, *Agger* 1971) The excretion of ' total gonadotrophins was within the normal range in all but a few of the men in the present material. Such biological assays may however not reveal minor deviations from normal levels A study of plasma FSH and plasma LH by radioimmunoassay in infertile men indicated that values of plasma FSH were increased in some patients who by bioassay showed a normal urinary excretion of total gonadotrophins (*Skakkebæk et al*, unpublished) The interaction between the gonads and the gonadotrophins is still a controversial subject (*Steinberger* 1971) No correlation was found between the values of the fractional 17 keto steroids and other features of the patients Almost all values were within normal limits and the few abnormal values showed no positive correlation of any type or degree of impairment of the seminiferous epithelium

Small testicular size (volume < 15 cm^3) was observed in six patients Although the present material constitutes a selected group of infertile men it is evident that measurement of testicular size is of reduced diagnostic value except in cases with extremely small testes *e g* patients with Klinefelter s syndrome

No results of studies on Leydig cells are presented Recent studies have revealed that estimates on Leydig cell volumes apparently are of little or no value if they are not based on a quantitative measurement (*Amad et al* 1969 *Heller et al* 1971) Furthermore in some of the cases there may be changes of the tubular membranes The aim of the present study was to reveal changes of the germ cells and the method of preparation used is not suitable for any detailed study of changes [illegible] tubular membranes For this reason no [illegible]tematic recording on fibrosis or hyalinization

tion was made It should be *noted that* very severe changes of the seminiferous epithelium may be present without accompanying gross degeneration of the tubular membranes (cf Figures 3, 6 and 9)

Future Aspects in Investigation of Testicular Failure

It seems rational to select adequately large *groups* of patients with the same type of testicular lesion and to lend such groups to appropriate investigations We therefore intend to relate the various types of lesions of the seminiferous epithelium to such factors as the plasma level of FSH and LH, to the testicular steroidgenesis and to the morphology of the spermatozoa in semen Furthermore, we intend to evaluate the effect of potential drugs to improve spermatogenesis on each type of impairment of the seminiferous epithelium

---

We wish to thank Mrs *Annelise Persson* for technical assistance Mrs *B Albertsen* for secretarial assistance, actuary *V F Gjeddebæk* for his assistance with the statistical evaluation of the results and senior investigator *Mavis Rowley* for her criticism in the preparation of the manuscript

This work was supported by the *P Carl Petersen's Fund*

REFERENCES

Agger P Scrotal and testicular temperature Its relation to sperm count before and after operation for varicocele Fertil Steril *22* 286–297 1971

Amad K N Lennox B & Mack W S Estimation of the volume of Leydig cells in man Lancet *2* 461–464, 1969

Clermont Y The cycle of the seminiferous epithelium in man Amer J Anat *112* 35–45 1963

Hammen, R Impaired fertility in man with special reference to the male Munksgaard Copenhagen 1944

Heller C G & Clermont, Y Kinetics of the germinal epithelium in man Recent Prog Hormone Res *20* 545 575 *1964*

Heller, G V & Heller, C G Quantitation of normal and abnormal germinal cells following administration of clomiphene citrate in normal men J Clin Endocr *30* 196–207, *1970*

Heller, C G, Lalli, M F Pearson J E & Leach, D R A method for the quantification of Leydig cells in man J Reprod Fertil *25* 177–184, 1971

Hotchkiss, R S Infertility in the male In Urology (eds M F Campbell & J H Harrison W B Saunders, Philadelphia 1970

Johnsen, S G Fractionation of urinary 17 ketosteroids I A simplified method for the chromatographic separation of urinary androgen metabolits Acta Endocr *21* 127–145 1956 a

Johnsen, S G Fractionation of urinary 17 ketosteroids II *Normal values for* men and women at different ages Acta Endocr *21* 146–156 1956 b

Johnsen, S G A clinical routine method for the quantitative determination of gonadotrophins *in 24 hour* urine samples Acta Endocr *28* 69 88 1958

Johnsen, S G A clinical routine method for the quantitative determination of gonadotrophins in 24 hour urine samples II Normal values for men and women at all age groups from prepuberty to senescence Acta Endocr *31* 209 227 1959

Johnsen S G Testicular biopsy score count — A method for registration of spermatogenesis in human testes Normal values and results in 335 hypogonadal males Hormones *1* 1 24 1970

Johnsen S G Evaluation of gonadotrophin analyses in male hypogonadism Acta Endocr 67 756–766 1971

Johnsen, S G Christiansen P Frandsen V A, Froland A & Nielsen, J Analysis of a dexamethasone/chorionic gonadotrophin test of adreno gonadol function in normal men as compared with dexamethasone test in normal women Acta Endocr *26* 587 605 1971

Paulsen, C A The testes In Textbook of Endocrinology (ed R H Williams) W B Saunders Philadelphia *1968*

Philip, J Skakkebæk N E, Hammen R Johnsen S G & Rebbe H Cytogenetic investigations in male infertility Acta Obstet Gynec Scand *49* 235 237 1970

Roosen Runge E C Quantitative investigations on human testicular biopsies I Normal testis Fertil Steril *7* 251 261 1956

Roosen Runge E C, Marburger E & Nelson W O Quantitative investigations on human testicular biopsies II Infertility and other conditions Fertil Steril *8* 203 219, 1957

Rowley M J & Heller C G The testicular biopsy Surgical procedure fixation and staining technics Fertil Steril *17* 177 186 1966

Rowley M J & Heller C G Quantitation of

the cells of the seminiferous epithelium of the human testis employing the Sertoli cell as a constant. Z. Zellforsch. *115* 461–472, 1971

Skakkebæk V E. Two types of tubules containing only Sertoli cells in adults with Klinefelter's syndrome. Nature 223 643–645 1969

Skakkebæk, V E. Abnormal morphology of germ cells in two infertile men. Acta path microbiol scand Section A. 80 374–378 1972

Skakkebæk, V E & *Heller*, C G. Quantification of human seminiferous epithelium. I. Histological studies in 21 fertile men with normal chromosome complements. J Reprod Fertil 32 179–189 1973 a.

Skakkebæk V E, *Hultén*, M & *Philip*, J. Quantification of human seminiferous epithelium. IV. Histological studies in 17 men with numerical and structural autosomal aberrations. Acta path microbiol scand Sect A 81 112–124, 1973 b.

Skakkebæk, V E, *Philip*, J, *Mikkelsen*, M, *Hammen*, R, *Nielsen*, J, *Perboll*, O & *Yde*, H. Studies on spermatogenesis, meiotic chromosomes, and sperm morphology in two males with a 47,XXY chromosome complement. Fertil Steril 21 645–656, 1970

Steinberger, E. Hormonal control of mammalian spermatogenesis. Physiol Rev 51 1–22, 1971

Steinberger, E & *Tjioe*, D I. A method for quantitative analysis of human seminiferous epithelium. Fertil Steril 19 960–970, 1968

Tillinger K G. Testicular morphology. A histo-pathological study with special reference to biopsy findings in hypogonadism with mainly endocrine disorders and in gynecomastia. Acta Endocr suppl 30 24 1–192 1957

TABLE 2 *Features of the Material*

Case	Age years§	Sperm ave mill/ml	Testis size* (cm³)	Secondary sex characteristics	Fathered children n
1	30	0 01	Small (7)	Normal	None
2	37	58 3	R Small (9) L Normal (22) o)	Normal	4 †
3	31	20 0	Normal (20)	Normal	None
4	19	21 0	Normal	Normal	None
5	34	0 6	R absent L Normal (35) o)	Normal	None
6	25	Not possible to obtain	Normal	Normal	2 "
7	17	—	Small	Normal	None
8	17	—			
9	21	—	Normal (18)	Normal	None
10	20	—	Normal (18)	Normal	None
11	22	—	Small L (6) R (10) o)	Normal	None
12	15	—	Normal	Normal	None
13	15	—	Small	Normal	None
14	39	—	Normal R (18) o) L (30)	Normal	None
15	19	–	R Normal (30) o) L Small (6)	Normal	None
16	25	47 0**	Normal (30)	Normal	None
17	19	Not possible to obtain	Normal	Gynecomastia	None

§ Age when the testicular biopsy was taken

* *Estimates of testicular size were obtained by palpation* The testicular size is shown in brackets in cases where an orchidometer was used o) indicates the side from which the biopsy originated in subjects with testes of unequal size

** Total volume was extremely low (0 5–0 8 ml semen)

† One abortion one normal boy (46 XY) one boy with Downs syndrome (47 XY + G) and one normal girl (45 XX D –G + t(Dq Gq))

" A boy with Down s syndrome (karyotype 46 XY 14 + t(14q 21q)) and a girl with cerebral malformation (karyotype 45,XX 14 21 + t(14q 21q))

in a randomly chosen section of the biopsy The estimates were expressed as a percentage of all tubules in the section Counts were only made on 30 cross sections of the category of tubules with most advanced spermatogenesis in these cases (only 14 and 18 tubules could be analysed in Case 8 and 14 respectively)

Semen analyses were performed according to the methods of *Hammen* (1944) and *Eliasson* (1971)

Data from 21 men with normal karyotypes normal sperm counts (> 60 mill/ml and no history of reproductive failure) was used as a control material (Table 3) (*Skakkebæk & Heller* 1973 a)

RESULTS

Germ Cells

Case 1, D/D translocation carrier The D/D translocation carrier showed a homogeneous histological picture with a normal number of spermatogonia and spermatocytes but an almost total lack of spermatids (Table 3, Fig 1 and 2 a)

Cases 2–6, 14/21 translocation carriers One of the five 14/21 translocation carriers (Case 2) showed a quantitatively normal spermatogenesis in the normal sized left testis (Table 3, Fig 2 b, 2 c and 3) No biopsy was taken from the right testis which was moderately reduced in size (8 cm³)

The biopsies from the remaining four patients all showed a normal or almost normal number of spermatogonia and spermatocytes, but a reduced number of spermatids (Table 3, Fig 2 d and 3) The biopsy from Case 3 contained a few obliterated tubules in addition to the general picture of a partial spermatogenic arrest (Table 4)

Case 7, patient with Down's syndrome and an unbalanced 13/21 translocation This case showed a homogeneous picture with normal spermatogenesis until primary spermatocyte level, but an almost total lack of spermatids (Table 3, Fig 4)

Cases 8–15, Down's syndrome with trisomy-21 A homogeneous histological picture was found in five of the eight cases with trisomy-21, i e Case 13 showed a pure Sertoli cell only picture, while Cases 9, 10, 12 and 15 had germ cells in all their tubules, although the number of these cells was severely reduced (Table 3, Fig 5 and 6 a) A partial Sertoli cell-only picture was seen in Cases 8 and 11 (Table 4) Case 8 demonstrated a severely reduced number of germ cells in the tubules with preserved spermatogenesis (Table 3), while there were too few tubules with germ cells for a sufficient analysis in Case 11 Case 14 showed a most heterogeneous picture with totally or partly obliterated tubules as well as tubules with arrest between the spermatocyte and the spermatid levels and also normal tubules (Tables 3 and 4)

Case 16 and 17, patients with an extra small centric chromosome fragment Biopsies from two patients with an extra small centric chromosome fragment showed pronounced inhibition of spermatogenesis A severe reduction in number of early and late spermatids was found in Case 16, while Case 17 showed a reduced number of all types of germ cells (Table 3, Fig 6 b and 7)

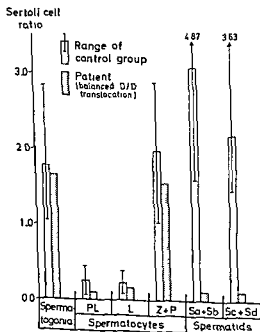


Fig 1 Sertoli cell ratio of different groups of germ cells in a D/D translocation carrier (Case 1) compared with values obtained from a study of a control group of fertile 46,XY men (*Skakkebæk & Heller*, 1973 a) The ranges of the control values are equivalent to estimates of the 2 5 and 97 5 percentiles of the frequency distribution for the fertile men. PL preleptotene, L leptotene, Z zygotene and P pachytene spermatocytes Sa, Sb, Sc and Sd are consecutive steps in the maturation process of spermatids

Leydig Cells

All biopsies appeared to have normal numbers of Leydig cells, and no Leydig cell nodules were found No actual counts were performed



Fig 2 a Section of testicular biopsy showing almost complete arrest of spermatogenesis between spermatocyte and spermatid level in a D/D translocation carrier (Case 1) × 130
b Normal spermatogenesis in a 14/21 translocation carrier (Case 2) × 130
c Same as Fig 2 b High power magnification × 500
d Abnormal spermatogenesis in a 14/21 translocation carrier Note the reduced number of spermatids (Case 3) × 500

TABLE 3 *Sertoli Cell Ratio and Total Sertoli Cell Number Obtained from Analysis of Biopsies from the Patients*

Case		Spermato-gonia	PL	L	Z+P	Sa+Sb	Sc+Sd	S
1		1 65	0 10	0 16	1 54	0 12	0 11	361
2		1 66	0 17	0 22	2 34	4 56	2 77	337
3 *		1 42	0 07	0 16	1 19	0 91	0 70	351
4		1 00	0 11	0 17	1 23	1 50	0 99	692
5		1 47	0 10	0 25	1 57	0 33	0 10	275
6		1 35	0 08	0 23	1 20	0 99	0 94	318
7		1 04	0 08	0 21	1 36	0 05	0 00	398
8 *		0 51	0 05	0 07	0 75	0 40	0 20	271
9		0 77	0 04	0 04	0 92	0 44	0 50	388
10		1 31	0 03	0 04	1 37	0 64	0 46	165
11				No count performed				
12		0 89	0 03	0 07	0 94	0 22	0 10	290
13				No count performed				
14 *		1 78	0 12	0 28	1 98	2 17	1 48	220
15		1 32	0 08	0 12	2 31	1 40	0 66	
16		1 45	0 13	0 26	1 69	0 43	0 39	422
17		0 51	0 02	0 10	0 31	0 13	0 08	527
Control	mean	1 77	0 25	0 22	1 96	3 05	2 14	
group	range	1 05	0 06	0 08	1 03	1 59	1 44	
		2 83	0 44	0 38	2 86	4 87	3 63	

PL = preleptotene, L = leptotene Z = zygotene and P – pachytene spermatocytes Sa, Sb, Sc and Sd are consecutive steps *in the* maturation process of spermatids S = Sertoli cells

* The results represent separate counts *on a* selected category of tubules (the category with most advanced spermatogeneses) Other categories of tubules were *also* present see Table 4

DISCUSSION

Three of the eight phenotypically normal subjects in the present study were ascertained through an infertility clinic one through presumptive gynecomastia and the remaining four through a chromosomally abnormal offspring One of the latter had a normal, while the rest had an impaired spermatogenesis These findings are in line with previous observations that defective spermatogenesis is often found in subjects with congenital chromosome abnormalities, but that some subjects have an apparently normal spermatogenesis despite the fact that they have an ab normal chromosome constitution (*Hultén & Lindsten* 1970, *Skakkebæk et al* 1973 b) Thus all infertile men should be cytogeneti

TABLE 4 *Distribution of Different Categories of Tubules in Biopsies with a Heterogeneous Picture*
Percentage of tubules showing

Case	Only Sertoli cells	Sertoli cells and spermatogonia	Sertoli cells spermatogonia and spermatocytes	All types of germ cells	Totally or partly obliterated tubules
3				91	9
8	23			77	
11	90			10	
14			11	44	45

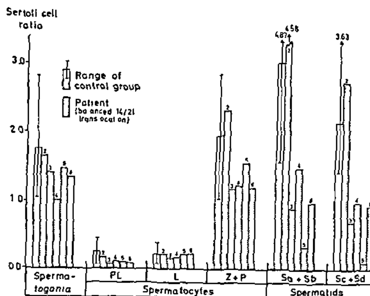


Fig 3 Sertoli cell ratio of different types of germ cells in five 14/21 translocation carriers (Cases 2, 3, 4, 5 and 6) compared with the range of the control group Note that Case 2 had quantitatively normal spermatogenesis

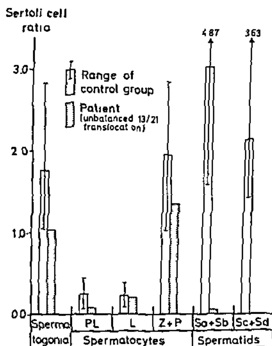


Fig 4 Sertoli cell ratio of different groups of germ cells in a patient with Down's syndrome due to an unbalanced 13/21 translocation (Case 7) Comparison with the range of the control group

cally analysed for diagnostic purposes even if they have a normal phenotype a normal semen analysis and/or a normal testicular histology The findings also show that testicular histology cannot be considered as a suitable indicator of a congenital chromosome abnormality

The histological changes observed in the testis of the eight phenotypically normal men consisted of a non-specific inhibition of spermatogenesis at all stages in analogy with previous studies (Table 5) The inhibition mainly involved the first spermatocyte level both in the D/D and one of the 14/21 translocation carriers and in one of the men with an extra, small marker chromosome In contrast, another 14/21 translocation carrier had an almost normal spermatogenesis, but with a reduced number of late spermatids Still another 14/21 translocation carrier had a completely normal histological picture in his normal sized left testis However the right testis, which was not studied, was smaller than normal, and might have had an abnormal

TABLE 5 *Literature Review Ascertainment and Testis Histology in Men with Structural Autosomal Aberrations or an Extra, Centric Small Fragment*

Reference	Chromosome aberration	Ascertainment Infertility	Other	Testis histology
Kjessler (1966)	D/D translocation carrier	x		*Partial spermatogenic arrest* at spermatocyte level
Hultén & Lindsten (1970)	–„–		x	Normal
Hultén & Lindsten (1970)	–„–		x	Decrease in all stages of spermatogenesis
Chandley (1970)	–„–			Reduced spermatogenic activity but all stages of maturation up to mature sperm
Mikkelsen (1966)	D/G translocation carrier		x	Normal (abnormal spermiogram)
Hultén & Lindsten (1970)	–„– a)		x	Normal (abnormal spermiogram)
Hultén & Lindsten (1970)	–„– a)	x		Reduced spermatogenesis and occasional sperm with pronounced degenerative changes
McIlree et al (1966 a)	21 22 ring chromosome	x		Partial Sertoli-cell only picture with depression of spermatogenesis
McIlree et al (1966 b)	Reduced short arm autosome 13 15	x		All tubules showed atrophy of germ cells, their only cellular content being Sertoli cells
Hultén et al (1966)	Extra centric small fragment a)		x	Some tubules contained a few spermatogonia only, but in other there were all stages up to spermatids and a few abnormal sperm.
Kjessler (1966)	–„–	x		*Sertoli-cell only picture*
Hultén & Lindsten (1970)	–„–		x	Peritubular fibrosis and a *considerable reduction of all* stages of spermiogenesis.
Chandley (1970)	–„–	x		Meiotic arrest between the first and second reduction divisions
Nielsen & Skakkebæk (to be published)	–„– a)		x	Severely reduced number of all types of spermatids.
Hultén & Lindsten (1970)	A/G translocation carrier		x	Normal

a) The patient is included in the present investigation

spermatogenesis The semen analysis correlated well with the histological findings in all cases studied in this respect.

Since carriers of the same type of translocation can have either an abnormal or a normal spermatogenesis it is difficult to draw any definite conclusions regarding the significance of these chromosome aberrations for the origin of the defective testicular function Furthermore, some of the men were ascer-

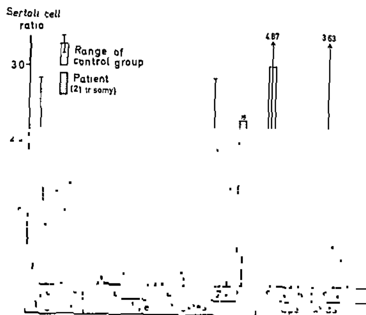


Fig 5 Sertoli cell ratio of different groups of germ cells in four patients with Down's syndrome (Trisomy 21) and only one category of tubules (Cases 9, 10, 12 and 15) Comparison with the range of the control group

tained through an infertility clinic and the findings of a chromosome aberration combined with impaired testicular function might therefore be fortuitous

The nine phenotypically abnormal patients with Down's syndrome all showed abnormalities of the seminiferous epithelium The one with an unbalanced 13/21 translocation had an almost total arrest at the primary spermatocyte level, and a wide range of abnormalities both between and within individuals were observed in the subjects with a 47,+21 chromosome constitution These abnormalities consisted of a complete Sertoli cell-only picture, a partial Sertoli cell-only picture and/or a "proportional" reduction in the number of all types of germ cells Approximately half of the tubules showed a quantitatively normal spermatogenesis in one case Previous investigations in males with Down's syndrome have also revealed an impaired spermatogenesis, but a normal or nearly normal histological picture has been reported in a couple of patients (Table 6) Whether this could be due to an undetected chromosome mosaicism is not known

Thus, specific histological abnormalities of the testis are only found in subjects with 47,XXY Klinefelter's syndrome and its variants (*Klinefelter et al* 1942, *Paulsen et al* 1968), while males with different types of autosomal aberrations and XYY males (*Hultén* 1970, *Skakkebæk et al*, 1973 b) demonstrate non-specific changes indistinguishable from those seen in subfertile men with normal karyotype (*Skakkebæk et al*, 1973 c)

The mechanism by which an abnormal chromosome constitution might affect spermatogenesis is difficult to evaluate, since for instance the same abnormal karyotype like trisomy-21 may be associated with a Sertoli cell-only picture, a "proportional" hypoplasia of all types of germ cells, an arrest at the first spermatocyte level as well as with an inhibition at the late spermatid level Even the pathogenesis of the specific testicular degeneration seen in 47,XXY Klinefelter's syndrome is not known (*Paulsen et al 1968*) *Ferguson-Smith* (1959) showed that prepubertal boys with this karyotype are lacking primordial germ cells in the majority of the tubules However, the onset of hyalinization on the



Fig 6 *a* Section of a biopsy from Case 9 (Down's syndrome Trisomy 21) Note the reduced number of germ cells although all types of cells are present × 130

b Section showing spermatogenesis in a patient with an extra fragment (Case 16) Note the severe reduction in number of spermatids and degenerated cells in lumen × 500

c Meiotic chromosomes in diakinesis of a D/G translocation carrier showing trivalent figure (T) and the XY bivalent (Case 2)

tubules occurs at puberty It must therefore be assumed that one or more pathological factors operate both during embryonic life and during puberty in this syndrome

There are theoretically several possible ways in which a chromosome aberration might interfere with the testicular development Thus the Sertoli cell only condition and the proportional hypoplasia may be due to a deficient migration of primordial

TABLE 6 *Literature Review Testis histology in Patients with Down's Syndrome*

Reference	Number of patients	Seminiferous epithelium
Mittwoch (1952) and *Miller et al* (1960)	5	Varying degree of spermatogenic arrest
Sasaki (1965)	1	Probably spermatogenic arrest
Finch et al (1966)	1	Spermatogenic arrest
Benda (1969)	3	Spermatogenic activity but no mature sperm
Benda (1969)	6	Sertoli cells but little or no spermatogenic tissue
Benda (1969)	17	Sertoli cells but no germ cells in 12 of 17 children with Down's syndrome
Hulten & Lindsten (1970)	2	Spermatogenic arrest in a D/G translocation mongol and all stages of spermatogenesis though considerably reduced in a patient with trisomy 21
Kjessler & de la Chapelle (1971)	2	Normal or near normal testicular histology with complete spermatogenesis some Sertoli cell-only tubules
Schroder et al (1971)	3	Complete spermatogenesis of lesser magnitude than in normal males

germ cells (*del Castillo et al* 1947) an arrest between the spermatocyte and spermatid levels may be associated with by degeneration

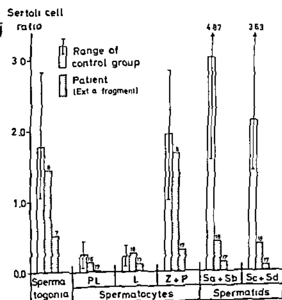


Fig 7 Sertoli cell ratio of different groups of germ cells in two patients with an extra fragment (*Cases* 18 and 19) compared with the range of the control group

during the meiotic divisions (*Nelson* 1950, *Kjessler* 1970) However it appears that many patients with the Sertoli cell only picture or spermatogenic arrest have normal karyotypes (*Kjessler* 1966 *Skakkebæk et al* 1973 c) The condition with partial impairment at the late spermatid level could be due to a selection against unbalanced gametes (*Kjessler* 1966) However, one patient with a balanced 14/14 translocation showed a normal spermatogenesis (*Hulten & Lindsten 1970 Caspersson et al* 1971), as did case 2 in the present study with a balanced 14/21 translocation These aberrations could be demonstrated in the germ cells (Fig 6 c) which makes it unlikely that the patients had a cell line with a normal chromosome complement in the testis which could explain the normal histological picture

All the changes in the germ cells might also be caused by for instance failure in the function of the Leydig or Sertoli cells or by an abnormal function in the gonadotrophic hormones Unfortunately very little is known about these factors for the development of

spermatogenesis although they are all probab ly of importance (*Steinberger* 1971)

We wish to thank Mrs. *Anneliese Persson* for technical assistance This work was supported by the *Danish Medical Research Council*, grant no 512 1390

REFERENCES

Benda C E Down s syndrome Grune & Stratten, New York 1969

Caspersson T Hultén M, Lindsten, J, Therkelsen, A J & Zech L Identification of different Robertsonian translocations in man by quinacrine mustard fluorescence analysis. Hereditas *67* 213 220 1971

del Castillo E G, Trabucco, A & de la Balze, F A Syndrome produced by absence of the germinal epithelium without impairment of the Sertoli or Leydig cells. J Clin Endocr *7* 493–502, 1947

Chandley A C In The Human Testis (eds *Rosemberg E & Paulsen C A*) Plenum Press N Y 1970

Eliasson R Standards for investigation of human semen Andrologie *3* 49 64 1971

Ferguson-Smith M A The prepubertal testicular lesion in chromatin positive Klinefelter s syndrome (primary micro-orchidism) as seen in mentally handicapped children Lancet *I* 219 222 1959

Finch R A Book J A Finley W H Finley S C & Tucker C C Meiosis in trisomic Down s syndrome Ala J Med Sci *3* 117 120 1966

Hammen R Impaired fertility in man with special reference to the male Munksgaard Copenhagen 1944

Hulten M Meiosis in XYY men Lancet *I* 717–718 1970

Hulten M Lindsten J, Fraccaro M Mannini, A & Tiepolo L Extra minute chromosome in somatic and germ line cells of the same person Lancet *2* 22 24 1966

Hulten M & Lindsten J The behaviour of structural aberrations at male meiosis Information from man In Human Population Cytogenetics Edinburgh 1970

Kjessler B Karyotype meiosis and spermatogenesis in a sample of men attending an infertility clinic S Karger Basel 1966

Kjessler B Meiotic studies in the human male In Modern trends in human genetics (ed A E H Emery) Butterworths London, 1970

Kjessler B Chromosomes and gametic output in 1000 infertile males (Abstract) Excerpt Med Int Congr Ser 234b 1971

Kjessler, B & de la Chapelle, A Meiosis and spermatogenesis in two postpubertal males with Down s syndrome 47,XY,G+ Clin Gen *2* 50–57, 1971

Klinefelter, H F, Reifenstein, E C & Albright, F Syndrome characterized by gynecomastia aspermia without a Leydigism, and increased excretion of follicle stimulating hormone J Clin Endocr *2* 615 627, 1942

McIlree M E, Tulloch, W S & Newsam, J E Studies on human meiotic chromosomes from testicular tissue Lancet *I* 679–682 1966 a

McIlree, M E, Price, W H, Court Brown, W M, Tulloch, W S, Newsam, J E & MacLean, N Chromosome studies on testicular cells from 50 subfertile men Lancet *2* 69 71, 1966 b

Mikkelsen, M Transmission of a 13–15/21 translocation in six families Ann Hum Genet *30* 147–161 1966

Miller, O J, Mittwoch, U & Penrose, L S Spermatogenesis in man with special reference to aneuploidy Heredity *14* 456 1960

Mittwoch U The chromosome complement in a mongolian imbecile Ann Eugen *17* 37–38 1952

Nelson W O Testicular morphology in eunuchoidal and infertile men Fertil Steril *I* 477–488 1950

Nielsen, J Friedrich U & Tsuboi, T Father and son with karyotype 47 XY,?Yq Humangenetik *11* 247–252, 1971

Nielsen J & Skakkebæk, N E Meiosis and testicular histology in a male with karyotype 47 XY,?Yq (To be published)

Paulsen C A Gordon, D L Carpenter R W, Gandy H M & Drucker W D Klinefelter s syndrome and its variants A hormonal and chromosomal study Recent Prog Hormone Res *24* 321 363, 1968

Philip, J Skakkebæk, N E Hammen R Johnsen S G & Rebbe H Cytogenetic investigations in male infertility Acta obstet gynec scand *49* 235–239 1970

Rowley, M J & Heller, C G The testicular biopsy Surgical procedure fixation and staining techn cs Fertil Steril *17* 177 186 1966

Sasaki M Meiosis in a male with Down s syndrome Chromosoma (Berl) *16* 652 657 1965

Schroder J Lydecker K & de la Chapelle A Meiosis and spermatogenesis in G trisomic males Humangenetik *13* 15–24 1971

Skakkebæk N E & Heller C G Quantification of human seminiferous epithelium. I Histological studies in twenty-one fertile men with normal chromosome complements J Reprod Fertil *32* 179 189 1973 a

Skakkebæk N E Hultén M Jacobsen P & Mikkelsen M Quantification of human seminiferous epithelium II Histological studies in

TABLE 6 *Literature Review Testis histology in Patients with Down's Syndrome*

Reference	Number of patients	Seminiferous epithelium
Mittwoch (1952) and *Miller et al* (1960)	5	Varying degree of spermatogenic arrest
Sasaki (1965)	1	Probably spermatogenic arrest
Finch et al (1966)	1	Spermatogenic arrest
Benda (1969)	3	Spermatogenic activity but no mature sperm
Benda (1969)	6	Sertoli cells but little or no spermatogenic tissue
Benda (1969)	17	Sertoli cells but no germ cells in 12 of 17 children with Down's syndrome
Hultén & Lindsten (1970)	2	Spermatogenic arrest in a D/G translocation mongol and all stages of spermatogenesis though considerably reduced in a patient with trisomy 21
Kjessler & de la Chapelle (1971)	2	Normal or near normal testicular histology with complete spermatogenesis, some Sertoli cell only tubules
Schröder et al (1971)	3	Complete spermatogenesis of lesser magnitude than in normal males

germ cells (*del Castillo et al* 1947) an arrest between the spermatocyte and spermatid levels may be associated with by degeneration

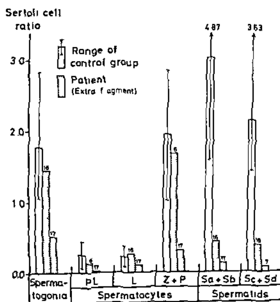


Fig 7 Sertoli cell ratio of different groups of germ cells in two patients with an extra fragment (Cases 18 and 19) compared with the range of the control group

during the meiotic divisions (*Nelson* 1950, *Kjessler* 1970) However, it appears that many patients with the Sertoli cell only picture or spermatogenic arrest have normal karyotypes (*Kjessler* 1966 *Skakkebæk et al* 1973c) The condition with partial impairment at the late spermatid level could be due to a selection against unbalanced gametes (*Kjessler* 1966) However, one patient with a balanced 14/14 translocation showed a normal spermatogenesis (*Hulten & Lindsten* 1970 *Caspersson et al* 1971), as did case 2 in the present study with a balanced 14/21 translocation These aberrations could be demonstrated in the germ cells (Fig 6c), which makes it unlikely that the patients had a cell line with a normal chromosome complement in the testis which could explain the normal histological picture

All the changes in the germ cells might also be caused by for instance failure in the function of the Leydig- or Sertoli cells, or by an abnormal function in the gonadotrophic hormones Unfortunately very little is known about these factors for the development of

Acta path microbiol scand Section A 81, 125–136, 1973

TRANSFER OF RABBIT UTERINE RENIN TO AUTOLOGOUS TISSUES PLACED IN CONTACT WITH THE UTERUS

PETER CLAES ESKILDSEN

The University Institute for Experimental Medicine (Head Jens Bing, M D), Copenhagen, Denmark

Loops of ureter, surgically embedded into the ipsilateral uterine horn of pregnant and non pregnant rabbits for 1 20 days, receive increasing amounts of renin, dependent on the renin level of the uterus Similar transfer of uterine renin occurs if the omentum majus or the abdominal wall are brought into contact with the uterus Interruption of the uterine contact for 4 days results in a decrease, but not in a disappearance, of renin from the ureter Application of Millipore filter tube around the ureter inhibits the passage of renin from the uterus in most cases, in a few cases, however, passage may still occur, even if the pore size is 0 5 μ Millipore diffusion chambers (pore size 0 1 μ–1 0 μ 14 μ) placed into pregnant uteri contain renin, independent of simultaneous penetration of cells These results show that *uterine renin is diffusible as extra cellular renin*, indicating an occurrence of considerable amounts of renin in the interstitial space of uterine tissue The possibility of a cellular renin formation in the ureter, induced by a special uterine factor or occurring on account of invasion of uterine capillaries containing renin producing cells, is discussed

The cellular source of the renin formation, demonstrated to take place in several mammalian organs is known in only a few organs

In the *kidney*, renin is almost exclusively located to the granulated epithelioid cells in the wall of the afferent arteriole, the cytoplasmic granules being the most probable site of renin storage (*Hartroft et al* 1964, *Faarup* 1968, *Hoffman & Hartroft* 1971 *Cook* 1971) In the *submaxillary glands* of different species of male albino mice, the large renin content is concentrated to the granulated ducts (*Bing & Faarup* 1965) where the renin similarly seems to be located to the granules (*Chiang et al* 1968) The occurrence of renin in human uterine tissue is predominantly localized to the chorion (*Skinner et al* 1968), but during *in vitro* conditions, cultures of myometrial tissue have also shown the ability to produce renin (*Symonds et al* 1968) The scattered distribution of Bowie-positive cells in the mesenchymal layer of the chorion and in the connective tissue of the myometrium is suggested (*Symonds et al* 1970) to represent the renin forming cells, although further evidence is lacking

In the *rabbit uterus*, it has not been possible to locate the renin formation to any granulated cells (*Bing & Faarup* 1966, *Eskildsen* 1971) Using microdissection on freeze dried, cryostat sections (*Eskildsen* 1972) it is shown that occurrence of considerable amounts of renin is connected with all structures of the uterine wall in the pregnant animal In the endometrium as well as in the myometrium, the highest renin content was found in the smaller vessels, not only in arterioles, but also in venoles and capillaries

Received 28 ix 72 Accepted 1 xi 72

Requests for reprints should be addressed to *Peter Claes Eskildsen*, The University Institute for Experimental Medicine, Norre Alle 71, 2100 Copenhagen Ø Denmark

Autotransplantation of uterine tissue to the anterior chamber of the eye was observed to result in an increased occurrence of renin in the surrounding iris tissue (*Eskildsen* 1971). This ability of uterine renin to pass from the uterus to the surroundings might be caused either by a passage of renin producing cells or by a diffusion of renin from the extracellular fluid.

In the present study, such passage of renin from the uterus to the omentum majus, the abdominal wall, and in particular to the ureter placed in close contact with the uterus, is exposed to further investigation. By means of Millipore filters with different pore size it is moreover attempted to evaluate whether or not this displacement of uterine renin is cellularly determined. The results contribute to the solution of the problem about the morphological state of the ubiquitous occurrence of renin.

MATERIALS AND METHODS

Sixty seven female albino country rabbits, Statens Seruminstitut, weighing 3–4 kg, were used. Fifty four were non pregnant and 13 were in the last week of pregnancy. The animals were divided into 5 groups. In *Group I* (17 animals), parts of the *omentum majus*, the *abdominal wall* and the *ureter* were placed in close contact with the uterine horn for 18–20 *days*. The intra uterine embedding of the omentum majus was performed on 2 pregnant animals, while fixation of the abdominal wall to the uterus was performed on 2 pregnant and 3 non pregnant rabbits. Three pregnant and 4 non pregnant animals were used for intra uterine embedding of a ureter loop. In 3 non pregnant animals a loop of the ureter was fixed along the outside of the uterine horn. In *Group II* (15 non pregnant animals, 8 were ipsilaterally nephrectomized), all animals were exposed to intra uterine embedding of the right *ureter* for *1–10 days*. In *Group III*, 7 non pregnant animals (3 of which were right sided nephrectomized) were treated as those in group II, but after 7 days (4 animals) or 10 days (3 animals) the right *ureter was detached from the uterus which was removed* while the *ureter was left intra abdominally* for an additional 4 days before it was removed. The former intra uterine segment of the ureter was marked by a silk suture. After ipsilateral nephrectomy of the animals in *Group IV*, a loop of the right ureter was covered by a tubular sheath (2 mm in diameter, 30 mm in length) of *Millipore filter* and placed within the uterus in 22 non pregnant animals for 1–2 days (9 animals) or for 10 days (13 animals). Three different filter types were used: 1) pore size 0.5 μ, thickness 125 μ, percentual porosity 80 per cent (CHOO20040 – 6 animals), 2) pore size 1.0 μ, thickness 130 μ, porosity 65 per cent (NRWPO4700 – 4 animals), 3) pore size 14 μ, thickness 130 μ, porosity 65 per cent (NCWPO4700 – 12 animals). The 0.5 μ pore filters were commercially available in the tubular form, but the tubes of the two other types were formed manually by means of the MF-MF cement substance (XX7000001). The filter sheath covered not only the intra uterine part of the ureter, but also 6–10 mm of the extra uterine parts proximally as well as distally (Fig. 2). *Group V* consisted of 6 pregnant animals which all had *Millipore diffusion chambers* placed within the uterus for 6 days before removal on the 28th day of pregnancy. The chambers were placed within the uterus through a 5–10 mm long antimesometrial incision between 2 amniotic sacs, 2–3 chambers per uterine horn. The diffusion chamber, consisting of a lucite

[illegible] XX [illegible] as previously described (*Vettesheim et al.* 1966). Prior to application, the chambers were filled with sterile Na K Ca Ringer solution through an opening in the ring. 3 types of filters with different pore size were used: 1) 0.1 μ (VCWPO1300 – 3 animals), 2) 1.0 μ (NRWPO1300 – 2 animals), 3) 14 μ (NCWPO1300 – 1 animal). The thickness (130–140 μ) and the percentual porosity (60–70 per cent) of the different types of filters were identical.

All *operations* were performed in pentobarbital sodium (40 mg/kg body weight) anaesthesia and the rabbits were treated with 250 000 I.U. penicillin per- and post-operatively. Tissue from the omentum majus, the abdominal wall and a part of the one ureter was placed in close contact with the uterine horn on one side by silk sutures (mersilene 4–0) allowing continuous blood supply to the intra uterine tissue.

Omentum majus: a piece of the distal free edge was placed in the uterus through a 10–20 mm long antimesometrial incision on the left uterine horn. The fat tissue was fixed to the uterus by sutures which simultaneously closed the uterine opening without further disturbance of the vascular supply to the intra uterine omental fat tissue.

Abdominal wall: the left uterine horn was thermocaustically separated from the mesometrium and the free mesometrial wall of the horn was fixed by sutures to the neighbouring peritoneal covered abdominal wall. As previously observed in the case of similar transposition of the uterine horn of the

guinea pig (*Bland* 1970), vascular connexion was established between the uterus and the abdominal wall after 2–3 weeks

Ureter the middle third of the right ureter was isolated intra peritoneally, released of fat in a length of 30–40 mm and part of it was placed *within the uterus through* a 20 mm long anti mesometrial incision on the right uterine horn After suture of the uterine opening the intra uterine loop of the ureter was still movable and in continued connexion with the extra uterine parts, proximally and distally In several cases (Group II and III), 2 loops of the ureter were placed within the uterus with an interjacent extra uterine loop of a length of 20–40 mm Four to six days later, hydronephrosis and hydro ureter of the proximal *extra uterine part* had developed on account of the uterine constriction of the ureter The intra uterine part seemed viable in all cases, but after 7 days it was *constricted, characterized* by *whitish fibrosis*, and had grown together with the endometrial surface of the uterine horn Even after 18–20 days, however, it was possible to separate the ureter from the uterus In cases where right sided nephrectomy was performed, the proximal half of the right ureter was drawn intra peritoneally, the free proximal end being fixed to the posterior abdominal wall In these cases the wall of the proximal extra uterine part of the ureter seemed to be haemorrhagic after 1–2 days and the artery was dilated by clotting or thrombosis These changes were not found in the intra uterine loop which was still supplied from the distally arriving artery

Blood samples (2 ml) were withdrawn from the ear artery of the conscious animals just before they were killed, and stored at 20° C as citrate plasma (50 µl 6 per cent sodium citrate per 1 ml of plasma)

Tissue samples were taken from the animals just after killing (by air embolism) The transplanted fat muscle and ureter tissues were carefully released from adhesive uterine tissue and, in the case of transposed ureter not only the intra uterine loop was sampled but also the extra uterine parts divided *in segments with a distance from the* uterus *of 1) 0–5 mm and 2) 5 10 mm*, proximally as well as distally (Fig 2) Before storing at 20° C, a small piece of every sample was taken for histological examination Normal untouched tissue was removed to serve as control in every experiment Also the *uterine segment exposed* to embedding of ureter and normal untouched segments from both horns were sampled

After careful cleaning of the filter surface the *diffusion chamber fluid* was collected with a micro pipette (Carlsberg), *immediately* centrifugated (1000 G) at room temperature, the supernatant from each chamber was used for renin determination while the sediment or fluid from the bottom of the tube was used for histological examination

Tissue extraction, homogenizing and transitory acidification to inhibit angiotensinase activity were performed as mentioned previously (*Eskildsen* 1970)

Renin assay was based upon the principle of determination of the decrease in angiotensinogen concentration in the course of time (*Poulsen* 1968) using radioimmuno assay for angiotensin I (*Eskildsen* 1972)

Renin concentration is expressed in *Goldblatt Units* per gram tissue (GU/g) by reference to a highly purified hog renin standard preparation, kindly supplied by *Dr Haas*, and identical with that donated by Dr Haas to the WHO Laboratory *for Biological Standards* (Nat Inst Med Res, Mill Hill, London) In the case of plasma renin determination, the integrated form of the Michaelis equation is used

$$ln \frac{s_o}{s_o - y} = k \quad E \quad t$$

in which s_o is the initial angiotensinogen (substrate) concentration, $s_o - y$ the angiotensinogen concentration at time t, k the rate constant, and E the enzyme concentration By addition of exogenous purified standard renin (*Poulsen* 1971) to the *plasma incubation the rate constant k is determined* 131 6 (S E 8 8, n – 5) ml/GU×hr, and the plasma renin concentration is then expressed in GU × 10^{-4} per ml The plasma renin substrate is indicated as nanograms angiotensin I per ml *plasma*

Antirenin assay was performed by means of an antirenin serum, kindly supplied by *Dr Poulsen*, and the method described by *Poulsen* (1966) was followed With a view to controle the binding *capacity of the antibody solution a highly purified* hog standard renin preparation diluted to renin concentrations (1 – 10 GU × 10^{-4}/ml) similar to those of the extracts was used

Histological examination was performed on tissue *fixed in Helly s solution for 24 hours, paraffin embedded*, cut in 4 micron thick sections and stained by haematoxylin eosin Van Gieson and PAS, cold procedure (*Petri* 1968)

In 2 cases of 10 days contact, the *vascular connexion between the uterus and the intra uterine* ureter loop was studied by intra aortic injection of an Indian ink solution (15 ml 5 ml of Indian ink (Pelikan) in 10 ml of saline) Prior to the injection the blood supply to the extra uterine parts of the ureter was carefully cut off

The *sediment of the diffusion chamber fluid*, or in the absence of sediment, the fluid from the bottom of the centrifugal tube was used for film preparations dried by air and exposed to May Grunwald Giemsa stains

RESULTS

1 *Renin Content of the Omentum, the Abdominal Wall and the Ureter after Surgically Produced Contact with the Uterus*

After 18–20 days' close contact with the *pregnant* uterus (Group I), the *renin concentration* of the exposed parts (Fig 1, open circles) of the *omentum majus* and the *abdominal wall* (consisting of the parietal layer of peritoneum, fascia and muscle layer) had markedly increased as compared with the values of the normal tissue (open triangles)

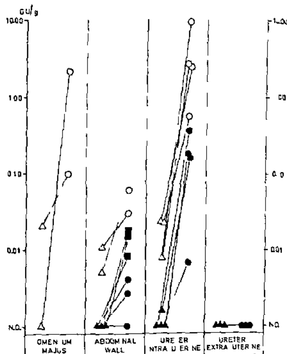


Fig 1 The renin concentration (GU/ g – logarithmic scale) of normal untouched tissue (triangles) and tissue exposed to close contact with the uterus (circles) during 18–20 days in pregnant (white symbols) and non pregnant (black symbols) animals In case of uterus abdominal wall contact the renin concentration of the complete layer of the wall (●) as well as of the connective tissue (fascia + peritoneum – ■) was determined in 3 non-pregnant animals Uterine contact with the ureter was performed partly by intraluminally embedding of the ureter (INTRA UTERINE) partly by fixation along the outher surface of the uterine horn (EXTRA UTERINE) Non-detectable values are placed at the level *N D* The lines connect the corresponding values from the individual rabbits

In the *non-pregnant* animals (Fig 1, black symbols), the renin concentration of the part of the abdominal wall which was connected with the uterus, showed only a small increase in 2 animals, being unmeasurable in the third If only the connective tissue layers, peritoneum and fascia (Fig 1, black squares), which were in closer contact with the uterus than the muscle layer, were analysed, markedly higher values of renin were found

As regards the renin concentration of the *normal, untouched tissue* it is of interest that, (in contrast to the unmeasurable values of the non-pregnant animals), not negligible concentrations of renin were measured in the omentum, the abdominal wall and the ureter of *terminally pregnant* animals

The *intra uterine* contact with the *ureter* resulted in a pronounced increase in the renin concentration of the exposed loops concentrations being much higher in the pregnant than in non-pregnant animals (Table 1), probably on account of the marked increase in uterine renin during pregnancy

After contact between the outside of the uterus and the ureter (Fig 1, Ureter Extra-Uterine) there was no effect on the renin content of the ureter This experiment further showed that the constriction with subsequent ischaemia of the exposed ureter-loop did not result in an increased renin content in the tissue

2 *Neutralization of the Renin-Like Activity of the Ureter and the Uterus by Antirenin*

The renin like substance in the *normal ureter* from pregnant rabbits and in *ureters exposed to uterine contact* from pregnant and non-pregnant animals was completely neutralized by anti hog renin serum The same effect of the antibody was observed after reaction with extracts of the *uterus* from pregnant and nonpregnant animals

ureter (< 0 0007 GU/g) In an attempt to eliminate the difference between the high values of the pregnant animals and the low ones of the non pregnant animals (Group I), the renin concentration of intra uterine as well as extra uterine parts of the ureter is expressed in per cent of the renin concentration of the exposed uterine horn (Fig 2) This procedure clearly shows that the level of the uterine renin concentration is responsible for the renin content of the ureter, since there is little difference between the percentual values of pregnant and non pregnant animals

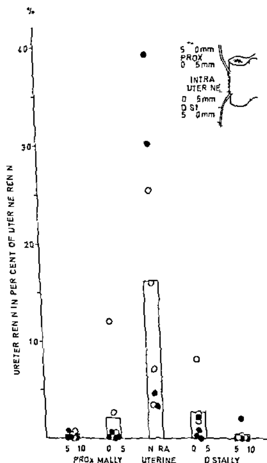


Fig 2 The distribution of renin in the intra uterine loop and in the extra uterine segments in 0–5 and 5–10 mm distance from the uterus in proximal and distal direction as showed in the diagram. The renin concentration of the ureter is expressed in per cent of that of the uterus. The means (columns) are calculated from the results of non pregnant (●) and pregnant (○) animals

3 *Distribution of Renin in Intra and Extra Uterine Parts of the Ureter Loop of Non Pregnant and Pregnant Rabbits*

In contrast to the high renin concentration of the intra uterine ureter loop, the renin concentration of the adjacent parts lying in proximal and distal direction outside the uterus showed a *steep fall* If the distance from the uterus increased from 0–5 mm up to 5–10 mm the renin concentration reached almost unmeasurable values, as found in the normal

4 *Renin Content of the Ureter after Short-Term Intra Uterine Embedding*

In order to study whether the increased renin content of the intra uterine ureter loop might be determined by an implantation of the ureter wall on the endometrial surface, the experiments were curtailed to a duration

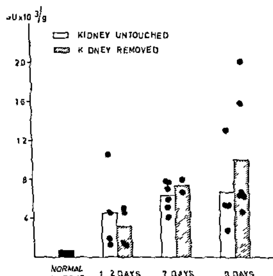


Fig 3 The renin concentration in GU × 10⁻³/ g of ureter after intra uterine embedding for 1 2 days 7 days or 10 days in animals with the ipsilateral kidney untouched (white columns) or removed (hatched columns) The individual values as well as the means (columns) are presented The non-detectable renin concentration, < 0 7 × 10⁻³ GU/ g of the untouched normal ureter from every animal is shown in the black column to the left

of 1–2 days, 7 days and 10 days in 15 non pregnant animals (Group II) Both in the case of the untouched kidney (*white columns*) and after ipsilateral nephrectomy (hatched columns), the renin concentration of the exposed ureter loop (Fig 3) was found to be markedly elevated being above the concentration of the normal ureter (< 0.7 GU $\times 10^{-3}$/g) after only 1–2 days intra uterine embedding If the duration of the experiment was extented to cover 7 days and 10 days the values showed an increasing tendency

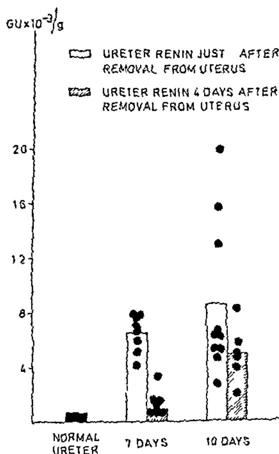


Fig 4 The renin concentration GU $\times 10^{-3}$/g in ureter loops which had primarily been placed in the uterus (7 or 10 days) but subsequently kept intra abdominally for 4 days without contact with the uterus (hatched columns) By way of comparison the renin concentration of ureter loops removed immediately after 7 or 10 days intra uterine embedding is shown (white columns) The level of renin concentration of the normal ureter is indicated by the black column to the left

5 *Presence of Renin in the Ureter after Discontinuation of the Uterine Contact*

The nature of the uterine induced occurrence of renin in the ureter loops was studied in 7 non pregnant animals (Group III) in which the ureter after 7–10 days intra uterine embedding was left intra abdominally for 4 days after total hysterectomy (Fig 4) The renin concentration was then determined in the ureter loops previously placed in the uterus When the intra uterine embedding of ureter had lasted for *7 days* (4 animals), the following period of 4 days without uterine contact resulted in a renin concentration (hatched columns) distinctly lower than that found immediately after 7 days intra uterine embedding (white columns) (Group *II*) This decrease in renin content of the ureter was uninfluenced by previous nephrectomy In the case of *10 days'* intra uterine embedding the renin concentration of the ureter after a further 4 days showed a smaller decrease in comparison with the renin concentration of ureters (Group II) not exposed to such an extra uterine period (Table 1)

6 *Presence of Renin in Intra Uterine Placed Ureter Covered by Millipore Filter*

In an attempt to investigate whether the passage of renin from the uterus to the ureter was determined by a passage of cells *Millipore filters* with different pore sizes (14 μ 1 0 μ and 0 1 μ) were placed around the ureter after ipsilateral nephrectomy in 22 non pregnant animals (Group IV)

If the uterine contact lasted for *1 2 days* the use of filters with large (14 μ) or small (1 μ) pores did not change the average ureter renin from that seen in controls without filters (Fig 5) In one case the use of filter with 14 μ pores resulted certainly in an extra ordinarily high renin concentration (22 0 GU $\times 10^{-3}$/g) in the ureter but in three other cases the same filter completely inhibited the passage of renin

Extension of the experiment to *10 days* however resulted in a marked difference between the increased renin concentration of

TABLE 1 *Survey of the Experimental Results*

Sample	Exp Group	Fig	n	mean	range	S D
Uterus						
Untouched tissue	II–IV	7	27	*0.315*	0.020 –1.008	±0.259
Ureter-exposed tissue	II–IV	7	26	*0.740*	0.221 –1.860	±0.405
Pregnant tissue	V		6	*8.58*	2.98 –23.78	
Ureter						
Left untouched	II IV	3–5	41		<0.0005 0.0008	
Right intra uterine embedding for						
1 2 days	II	3	8	*0.0038*	0.0013–0.0106	
7 days	II	3	8	*0.0066*	0.0042–0.0080	
7 days + 4 days outside uterus	III	4	8	*0.0014*	0.0007–0.0034	
10 days	II	3	10	*0.0087*	0.0028–0.0201	
10 days + 4 days outside uterus	III	4	6	*0.0050*	0.0021–0.0084	
18 days	I	1	4	*0.183*	0.0067–0.3726	
18 days during pregnancy	I	1	4	*3.868*	0.566 –9.700	
Ureter + Millipore Filter						
0.5 1.0 μ pores (1 – 10 days)	IV	5	4		<0.0009	
			6	*0.0029*	0.0012–0.0063	
14 μ pores (1 10 days)	IV	5	5		<0.0009	
			7	*0.0053*	0.0020–0.0220	
Millipore Diffusion Chambers in Pregnant Uterus						
14 μ pores	V	6	4	*0.077*	0.048 –0.068	
1 μ pores	V	6	13	*0.127*	0.035 –0.330	
0.1 μ pores	V	6	12	*0.101*	0.025 –0.268	
Plasma Renin Concentration						
Normal animals			18	*0.92 × 10⁻⁴*	0.34 2.44 × 10⁻⁴	±0.46 × 10⁻⁴
Experimental animals	II IV		33	*1.10 × 10⁻⁴*	0.18 1.95 × 10⁻⁴	±0.50 × 10⁻⁴

The renin concentration is expressed in *Goldblatt Units* per gram or per ml (in the cases of diffusion chamber fluid and plasma) If not otherwise stated the samples belong to non pregnant animals

the controls and the unchanged, low or unmeasurable values of the filter covered ureters No evident difference was observed whether the pore size of the filters was 14 μ or 0.5 μ Since the filter tubes in these experiments were covered by a thin layer of connective tissue the passage of renin was possibly inhibited by clogging up of the pores

7 Passage of Renin through Diffusion Chambers

Millipore diffusion chambers were placed within the uterus of 6 *pregnant* rabbits (Group V) in the last week of pregnancy After removal 6 days later the chamber fluid was studied for the presence of renin and cells Application of chambers with pores of 14 μ (1 animal) and even of 1 μ (2 animals) resulted in passage of considerable amounts of renin (Fig 6) as well as numerous cells mainly neutrophile leucocytes but also some erythrocytes and a few eosinophile and basophile leucocytes

If the pore size was decreased to 0.1 μ no cells or sediment was observed in the chamber fluid which, however contained about the same concentration of renin as the other chambers

The renin concentration of the supernatant of *amniotic fluid* was analysed in 4 of the

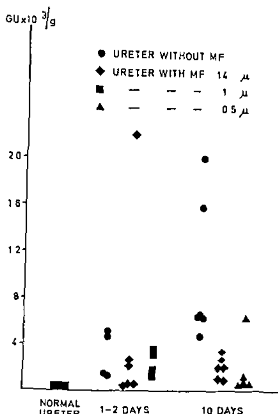


Fig 5 The renin concentration of ureter covered by tubular Millipore filter during 1–2 days or 10 days' intra uterine embedding In each case the influence of the pore size, 14 μ (◆), 1 μ (■), or 0 1 μ (▲) of the filters is studied and compared with the renin concentration of non filter covered intra uterine segments (●)

animals, the low values, 0 001 – 0 005 GU/ml, excluded that the renin content of the diffusion chambers had derived from the amniotic fluid

8 *Renin in Uterine Segments Exposed to Embedding of Ureter*

In comparison with segments of the untouched uterine horn many uterine segments exposed to ureter embedding of short duration showed a pronounced elevation of the renin content, while in other cases only a smaller or no increase was found In Fig 7, the 26 studied cases are seen to be classified into 3 groups according to the renin level of the normal uterine horn 1) 0 02 – 0 10 GU/g, 2) 0 10 – 0 30 GU/g, 3) 0 30 – 1 00 GU/g It is obvious that the ureter embedding results in an especially pronounced increase of the local uterine renin concentration in group 1), the ratio between the 2 average values being 1 8 8 If the renin concentration of the normal uterine tissue is increasing the renin activating effect of ureter-embedding decreases, the ratio between the average values being 1 5 in group 2), and 1 1 5 in group 3)

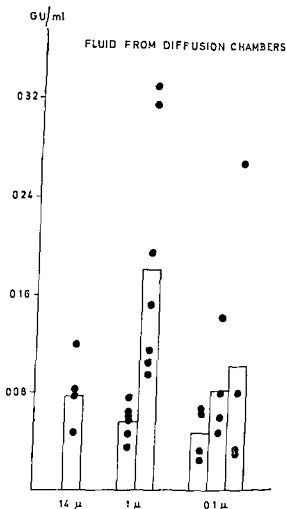


Fig 6 The renin concentration GU/ml, of the fluid from intra uterine placed Millipore diffusion chambers in 6 pregnant animals The influence of the pore size of the filters, 14 μ (1 animal), 1 μ (2 animals) 0 1 μ (3 animals), on the renin content is demonstrated The renin concentration of the single chamber fluid is estimated (●) and the means are calculated (columns) in every animal

All groups considered together (Table 1), the renin concentrations of the ureter exposed uterine segments were significantly higher ($p < 0.0001$) than those of the normal, left uterine horn This influence on uterine renin was limited to the uterine segments in which the ureter was embedded, as the renin concentration of untouched segments from the same uterine horn showed only small difference from that of the normal uterine horn

9 *Histological Examination*

Ureter The morphological changes of the intra-uterine placed ureter were most pronounced in the peripheral *adventitial layer* which after 1–2 days' uterine exposure was markedly enlarged with dilated, erythrocyte stuffed vessels and an extravascular occurrence of numerous granulocytes and mononuclear cells After 7 days, the fat cells in this layer were replaced by deposits of a structureless fibrinoid substance and later on (10 days) by collagen fibres and fibroblasts The vascularization was still well-developed and many leucocytes were placed in groups or dispersed After 18–20 days, the adventitia was shrunken, mainly consisting of collagen fibres with only few cells and smaller vessels

The *muscular layer* was in 1–10-day-experiments markedly distended with increasing amounts of collagen substance and fibres, and after 18–20 days it was thin, contracted with strands of connective tissue

The transitional *epithelium* of the *mucous membrane* looked mainly unchanged, while the *lamina propria* was characterized by oedema and vasodilatation in the short term experiments, but by fibrosis in the experiments of 18–20 days' duration

The presence or absence of the kidney was without influence on these morphological changes, and application of Millipore filter around the ureter did not seem to change the picture If the uterine contact was interrupted during the last 4 days of the experiment, the ureter tissue seemed to be more damaged, presenting cell atrophy, necrosis and, later, fibrotic changes

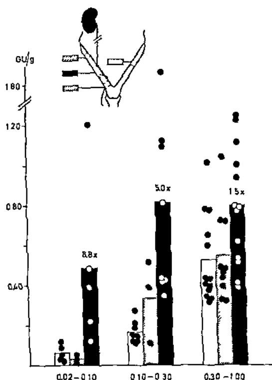


Fig 7 The renin concentration, GU/ g, of the ureter exposed uterine segments (black columns) compared with that of untouched segments from the same horn (hatched columns) and that of tissue from the normal, untouched uterine horn (white columns) in each of 26 non pregnant animals, all used for 1 10 days' ureter embedding The individual values are divided into 3 groups according to the renin level of the normal uterine horn 1) 0 02–0 10, 2) 0 10–0 30, 3) 0 30–1 00 GU/ g The numbers above the black columns indicate the increase, in folds (x), above the values of the white columns

By *intra-aortical injection of Indian ink* in 2 experiments of 10 days' duration it was seen (after ligation of the extra-uterine vascular contribution) that the contact between uterus and ureter included vascular connection

Uterus The *uterine endometrium* exposed to contact with the ureter was characterized by dilated, blood filled vessels, and the distance between the cell nuclei was increased in the stroma, probably due to increased water imbibition The number of eosinophilic leucocytes was greatly enlarged The epithelium of the surface as well as the glands and the myometrium appeared normal

10 *Plasma Renin and Substrate Concentrations*

By way of comparison of the *plasma renin concentration* of arterial blood from normal animals and that of animals exposed to the ureter experiments of 1–10 days' duration (Table 1) it was found that no significant difference was present ($p > 0.50$) The plasma renin *substrate concentration* (612 ± 280 (S D) ng/ml) however was significantly elevated ($p < 0.001$) above the normal substrate concentration (338 ± 95 (S D) ng/ml)

This finding is in agreement with the adrenal dependent increase in renin substrate observed after injuries leading to stress induced ACTH release (for literature see *Bing* 1972)

The majority (25) of the experimental animals were unilaterally nephrectomized but the values from these animals were not different from those of non nephrectomized animals

DISCUSSION

The present study demonstrates that contact between uterine tissue and different types of autologous tissue such as the omentum majus, the abdominal wall and the ureter results in an increased concentration of renin in these tissues After embedding of a loop of the *ureter* into the uterus the renin content in the intra uterine loop is found to be much higher than that in the extra uterine parts showing that the renin is of uterine origin Ipsilateral *nephrectomy* is without any influence while *pregnancy* on account to the elevated uterine renin concentration results in a markedly increased transfer of uterine renin

The *duration* of the contact between uterus and ureter is important as extension of the experiment from 1 to 7–10 days and further to 20 days is followed by a pronounced increase of the renin content of the exposed ureter loop After 10 days contact the 2 tissues are closely adhesive and *vascular connexion* is demonstrated The finding of small but clearly elevated renin concentrations even after 1–2 days contact with the uterus indicates however that vascular connexion is not a condition of a renin transfer

Interruption of the contact with uterine tissue leads in the course of 4 days to a decrease of the renin concentration of the exposed ureter loop This decrease is less pronounced 10 days after preceding uterine contact than 7 days after such contact but the renin concentration is still elevated in all cases It seems probable that the rate of disappearance of renin is prolonged in the damaged tissue of the ureter but even so it is questionable whether this occurrence of renin is explained only by the presence of extra cellular renin

In the study of a possible passage of renin containing cells from the uterus to the ureter the *Millipore filters* were used While a 1 2 day application of filter tubes with pores from 14 to 0 5 μ around the ureter does not result in an obvious inhibition of the passage of uterine renin a 10 day application clearly inhibits a further increase of renin Some clogging up of the pores is possibly taking place especially during the period of 10 days where connective tissue was seen to cover the outside of the filter tubes This is probably the reason why values were low in these experiments

By means of *Millipore diffusion chambers* placed into terminally pregnant uterine horns it is demonstrated that considerable and equal amounts of renin penetrate into the chamber whether a cell passage is in evidence (14 μ and 1 μ pores) or eliminated (0 1 μ pores) This finding shows that *uterine renin* occurs in considerable concentrations in the *extra cellular space* of the uterus which allows the renin to *diffuse* into foreign tissues or diffusion chambers embedded in the uterus

Nevertheless it is questionable whether diffusion of renin is the only way of transfer of uterine renin to other tissues brought in contact with the uterus

The transfer of a *uterine factor inducing renin formation* in the cells of the embedded tissue is one possibility The presence of such a factor could explain the finding of elevated

renin concentrations in the normal ureter the omentum and the abdominal wall of terminally pregnant animals on condition that the factor was demonstrable in the blood during pregnancy This is a purely theoretical possibility, however, since such a factor so far remains to be discovered

A *capillary ingrowth including renin producing cells* from the uterus to the ureter is another possibility which is supported by the demonstration of a vascular connexion between the uterus and the ureter at the time at which the ureter renin is increasing The demonstration of an elevated renin concentration in the *ureter exposed uterine segment*, characterized by morphological signs of increased vascular activity, may furthermore be in support of the postulation that there is a connection between the capillary – venole system and the renin producing cells in the uterus

Studies of the microcirculation in *skin autografts* in rats (*Marckmann* 1966) and in *granulation tissue* of ear chambers in rabbits (*Lindhe & Brånemark* 1970) have clearly demonstrated that proliferation of capillaries from venoles in the periphery into the graft and the granulation tissue takes place in the course of 4–7 days. In *skin wounds* in rabbits (*Myers & Cherry* 1971) newly formed vessels are likewise seen to cross the suture line about 3–6 days after the skin transection In studies of *tissue explants* in diffusion chambers (*Aloisi et al* 1970) such proliferation and formation of new capillaries is found to occur in omentum muscle tissue connective tissue and subcutaneous tissue

Although these investigations thus support the possibility of a vascular proliferation from the uterus into the ureter the results of the present study do not allow any conclusions to be drawn about a connexion between a capillary ingrowth and the occurrence of renin in the ureter embedded in the uterus Further studies are needed

This work was supported by grants from *Nordisk Insulinfond* and the *Legacy of Miss P A Brandt*

REFERENCES

1 *Aloisi, M Giacomin, C & Tessari R* Growth of elementary blood vessels in diffusion chambers I Process of formation and conditioning factors Virchows Arch Abt B Zell path 6 350–364 1970

2 *Bing, J* Relation between renin substrate and acute phase proteins Studies of normal and adrenalectomized rats Acta path microbiol scand Section A 80 646–650, 1972

3 *Bing, J & Faarup, P* Location of renin (or a renin like substance) in the submaxillary glands of albino mice Acta path microbiol scand 64 203–212 1965

4 *Bing, J & Faarup P* A qualitative and a quantitative study of renin in different layers of the rabbit uterus Acta path microbiol scand 67 169 179, 1966

5 *Bland K P* Uterine autotransplantation to the abdominal wall in the guinea pig J Endocr

6 *Chian*
& Co
of granules containing ren n and kallikrein Circ. Res 23 507 517 1968

7 *Cook W F* Cellular location of renin In Fisher J W (Ed) Kidney Hormones, 1 ed Academic Press New York and London, 1971, p 122 127

8 *Eskildsen P C* Place of renin formation in rabbit uterus Acta path microbiol scand 78 A 473–481 1970

9 *Eskildsen P C* Place of renin formation in rabbit uterus II – Renin in intraocular endometrial and myometrial transplants Acta path microbiol scand 79 A 123 133 1971

10 *Eskildsen P C* Location of renin in rabbit uterus by help of microdissection Acta path microbiol scand 80 A 241 252 1972

11 *Faarup P* Ren n locat on in the different parts of the juxtaglomerular apparatus in cat kidney – 2 Fractions of the afferent arteriole the cell group of Goormaghtigh the efferent

12

renin Further stud es with the fluorescent antibody technique Can Med Ass J 90 163–178 1964

13 *Hoffman N I & Hartroft P M* Application of peroxidaselabelled antibodies to the localization of renin J Histochem Cytochem 19 811 813 1971

14 *Lindhe J & Brånemark P I* Observations on vascular proliferation in a granulation tissue J periodont Res 5 271 292 1970

15 *Marckmann A* Reaction of skin to autotransplantation Studies on the microcircula

tion and the biochemical composition in dermis Thesis Munksgaard, Copenhagen 1966, p 32–39

16 *Myers, M B* & *Cherry, G* Rate of revascularization in primary and disrupted wounds Surg, Gynec & Obstet *132* 1005 1008, 1971

17 *Nettesheim, P, Makinodan, T* & *Chadwick, C J* Improved diffusion chamber cultures for cytokinetic analysis of antibody response Immunology *11* 427–439 1966

18 *Petri, M* A routine counterstain for the PAS reaction Acta path microbiol scand *73* 303, 1968

19 *Poulsen, K* Kinetic and measurement of the renin – antirenin system. Acta path microbiol scand *68* 197–205, 1966

20 *Poulsen, K* Measurement of renin in human plasma Scand J clin Lab Invest *21* 49–61, 1968

21 *Poulsen, K* No evidence of active renin – inhibitors in plasma The kinetics of the reaction between renin and substrate in non pre treated plasma Scand J clin Lab Invest *27* 37–46, 1971

22 *Skinner, S L, Lumbers, E R* & *Symonds E M* Renin concentration in human foetal and maternal tissues Am J Obst & Gynaec *101* 529–533, 1968

23 *Symonds, E M, Stanley, M A* & *Skinner S L* Production of renin by *in vitro* cultures of human chorion and uterine muscle Nature *217* 1152 1153, 1968

24 *Symonds, E M, Skinner, S L, Stanley, M A, Kirkland, J 1* & *Ellis, R C* An investigation of the cellular source of renin in human chorion J Obst & Gynaec Brit Cwlth *77* 885–890, 1970

Acta path microbiol scand Section A 81, 137 144, 1973

MALIGNANT GIANT-CELL TUMOUR OF THE COLON

KWAME ESHUN WILSON

Pathology Institute, Odense University Hospital, Odense, Denmark

A case of a giant cell tumour of colon histologically indistinguishable from giant cell tumours of bone is reported The tumour consisted of multinucleated osteoclast like giant cells and intervening spindle cells the latter exhibiting pleomorphism and mitotic activity, indicating malignancy The writer is unware of a similar case

The extraskeletal malignant giant cell tumour is recognized by the World Health Organization s literature on tumour classifications under the heading of tumour of disputed or uncertain histogenesis (15) Giant cell tumour is by no means an uncommon tumour of the bone. While giant cells may be found in various soft tissue tumours, giant cell tumours with histological features indistinguishable from those of tumours that occur in bone are uncommon outside the skeletal system Most have been described in tendon sheaths and fascial structures of the thigh (6), but more rare localizations like the thyroid (9), heart (2) pancreas (10), skin (1), the abdominal wall (4), and breast (3) have also been reported In view of recent literature, indicating that extraskeletal giant cell tumours may be of epithelial origin, the present case of a giant cell tumour of the colon indistinguishable from giant cell tumour of the bone is considered of interest

MATERIAL AND METHODS

A 44 year-old woman was admitted with the main complaint of constant abdominal pains of three months duration Six years previously resection of both ovaries was performed with removal of two chocolate cysts and an X ray examination at that time revealed colitis coli descendentis et sigmoidei, but no sign of tumour Prior to the present admission she had noticed blood and mucus in the stools and she could feel a tumour mass in the left side of the abdomen Clinical examination showed a palpable, hard and mobile tumour localized in the left upper quadrant of the abdomen Barium enema showed a 14 cm long irregular, stiff stricture of the descending colon A laparotomy was done and 15 cm of the colon descendens which was partly fixed to the surroundings was resected There were no peritoneal deposits and no liver metastases An end to-end anastomosis was performed No postoperative complications

The surgical specimen was a 14 cm long piece of large intestine with a centrally localized, 8×6




Fig 1 Surgical specimen of resected colon with centrally localized fungating tumour with ulceration

Received 4 ix.72 Accepted 29 ix 72

Requests for reprints should be addressed to *Kwame Eshun Wilson*, Odense University Hospital, DK 5000 Odense

Present address Path. Dept., Reg Hospital, Effia Nkwanta Hospital, Sekondi Takoradi, Ghana.

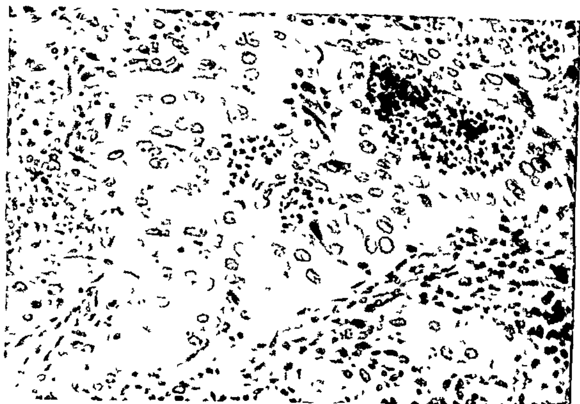


Fig 2 Photomicrograph of small area showing an irregular glandular structure (Haematoxylin and eosin *100* ×)

cm, well demarcated fungating tumour leaving 5 cm of the colonic circumference tumourfree The tumour was protruding about 4½ cm into the lumen almost occluding the latter The surface of the tumour showed a deep central ulceration (Fig 1) The tumour was firm but friable The cut surface showed homogenous greyish white tissue alternating with softer yellowish and brownish areas The tumour was up to 6 cm thick, infiltrating all the layers of the intestinal wall including adjacent mesenteric fatty tissue For light microscopic study, multiple sections were stained with haematoxylin eosin, PAS (with and without diastase digestion), Alcian Blue, Mayers mucicarmin, von Kossas method for calcium and ordinary stains for elastin, reticulin, iron and collagen Frozen sections of formalin fixed material were stained with oil Red 0 for neutral fat For electron microscopy study, small pieces of tissue which had been in formalin for several days were transferred to 3 per cent glutaraldehyde in cacodylate buffer pH 7 2 for 22 hours followed by washing in sucrose in the same buffer The tissue was cut in small pieces, the largest diameter measuring 1 mm fixed in 1 per cent OsO_4 phosphate buffer for 2 hours and after dehydration embedded in Epon One micron thick sections for orientation purposes, and thin sections for electron microscopy, were cut in a LKB III ultramicrotome Poststaining was done with Zinc uranyl acetate and lead hydroxide and the sections were examined in a Hitachi HS 8 electron microscope

RESULTS

The surface of the tumour consisted of a thin layer of necrotic tissue Tumour tissue replaced completely the mucosa, submucosa and the muscular coat and it infiltrated the adjacent muscular coat, the serosa and the surrounding fat tissue There was no connection between the tumour tissue and the crypts of the adjacent mucosa In two very small areas, at some distance from the ulcerated surface, irregular glandular structures were observed (Fig 2) Apart from this, multiple sections showed a strikingly uniform picture throughout without any resemblance to epithelial structures The tumour tissue consisted of a sparse stroma of reticular and collagen fibrils with two distinct cellular elements

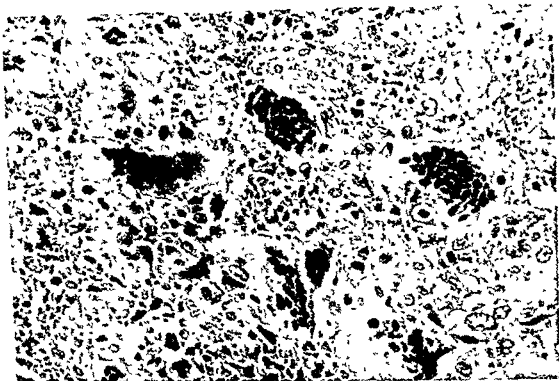


Fig 3 Photomicrograph showing the general appearance of the tumour The cytologic features and relationship between giant and stromal cells are markedly similar to those seen in giant-cell tumour of bone (Haematoxylin and eosin 100 ×)

(Fig 3) The first is made up of multinucleated giant-cells more or less evenly distributed throughout, and measuring about 70 to 90 microns in diameter, although some were much larger The cytoplasma of the multinucleated cells was abundant and eosinophilic They contained from 15 to 30 nuclei each The nuclei were uniform in size, shape and staining quality They were round to oval with diffusely distributed chromatin and no mitotic figures (Fig 4) The second type of cells were made up of closely packed oval or spindle shaped polymorphic cells. The cytoplasma was faintly eosinophilic The nuclei of these cells showed great variety of size, shape and staining quality and at least one mitotic figure was found in each high power field A few of these cells contained two or three nuclei (Fig 5) The tumour was rich in blood vessels and there were many areas with haemorrhage Diffuse infiltration of lymphocytes, plasmacells, haemosiderin-laden macrophages, foam-cells and especially eosinophilic granulocytes was observed No osteoid tissue, calcification, fungi or other pathological abnormality was seen

The results of the histochemical reactions were comparable to those described in giant-cell tumours of the bone (10) The cytoplasma of the giant-cells was stained, either diffusely or in a localized area with periodic acid-Schiff (even after diastase digestion) and rather slightly with Alcian Blue, but did not show so intense staining as epithelial mucin and the stromal cells were negative for all these stains.

Von Kossas staining for calcium was negative Mayers mucicarmine was also negative for mucin substances

However, the epithelial cells in the adenomatous structures were positive for Alcian Blue periodic acid-Schiff and mucicarmine

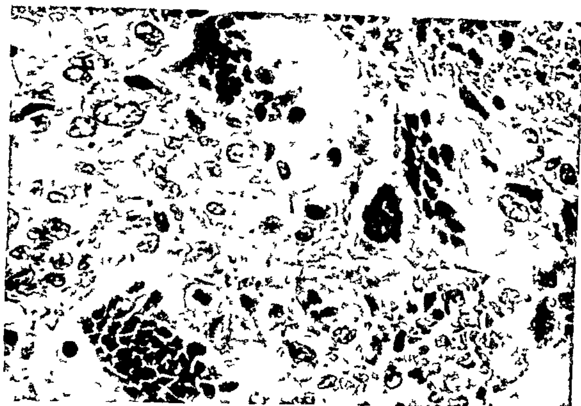


Fig 4 Photomicrograph of the same area showing as in Fig 3 (left) typical giant cells with numerous uniform nuclei (Haematoxylin and eosin 250 ×)

stains in the ordinary manner Frozen sections stained for fat and lipids were faintly positive

Electron Microscopy

The findings in the ultra thin Epon embedded sections confirmed the presence of mono and multinucleated cells both containing rather abundant rough endoplasmatic reticulum (Fig 6–7) The intercellular space was quite small or narrow finely fibrillar perhaps representing a condensation of glycocalyx Although some cells contained granules either like those of mast cells or those of eosinophilic cells other cells contained rather large round dark unidentified bodies perhaps lysosomes and vesicles (fig 8) but no granules were found in the channels of the endoplasmatic reticulum so there was no convincing feature suggestive that epithelial and specifically glandular elements were the cells of origin of the tumour in this case However this negative finding might be caused by lack of proper fixation method

DISCUSSION

The giant cells of the present tumour were osteoclast like The intervening spindle shaped and pleomorphic cells exhibited obvious neoplastic properties ruling out the possibility that the process was of inflammatory nature The histological picture was indistinguishable from that of a giant cell tumour of bone and corresponds exactly to that of an extraskeletal malignant giant cell tumour of soft parts illustrated in WHO's International Histological Classification of Tumours (15)

Fibroxanthoma (fibrous histiocytoma) (15) a benign often richly vascular growth made up of histiocytes and collagen producing fibroblast like-cells characteristically arrang

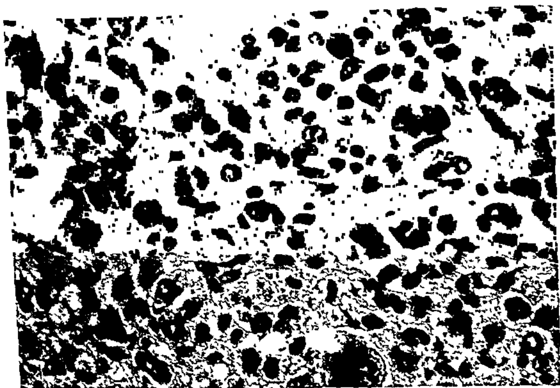


Fig 5 Photomicrograph of another area. Here the picture has the appearance of a spindle cell sarcoma The cells are polymorphic and the nuclei show great variation in size, shape and staining quality. (Haematoxylin and eosin 250 ×)

ed in a whirled or cartwheel pattern, often with lipid laden macrophages, could be well distinguished from this lesion The other variant termed atypical fibroxanthoma, dominated by cellular polymorphism with multinucleated giant cells with occasional Touton type has quite a distinct feature

Xanthoma, Xanthogranuloma (15) and especially Retroperitoneal Xanthogranuloma (Oberling) (8) with their characteristic lipidcarrying histiocytes, acute or chronic inflammatory elements, varying amounts of fibrous connective tissue, occasional Touton type of giant-cells and frequent but not often association with high serum cholesterol levels constituted a possible series of differential diagnoses

However, staining for haemosiderin and for fats and lipids were faintly positive in the present case and the giant cells were not of the Touton type Finally, the present tumour only contained very limited amounts of collagen It was therefore concluded that the present case was an extraskeletal giant cell tumour of soft parts with a highly unusual localization

The true nature of extraskeletal giant-cell tumours has been a matter of controversy In older literature (3) these tumours were considered to be of mesenchymal origin A pancreatic neoplasm which by light microscopy had an appearance indistinguishable from that of the giant-cell tumour of the bone has recently been reported (10). Electron microscopy revealed that both giant and stromal cells had abundant granular endoplasmatic reticulum containing intracisternal granules of proteic nature similar to those described in pancreatic acinic cells Microvilli were present in the giant-cells and numerous desmosomes were found between the stromal cells These features were interpreted as indicating that

Fig 6 Part of a giant cell with multinucleation and endoplasmatic reticulum (8,400 ×)

Fig 7. Some mononuclear tumour cells showing some endoplasmatic reticulum, small granules and fine threads in the cytoplasma The cell borders are surrounded by a zone of finely granular and moderately electron dense material (8,400 ×)

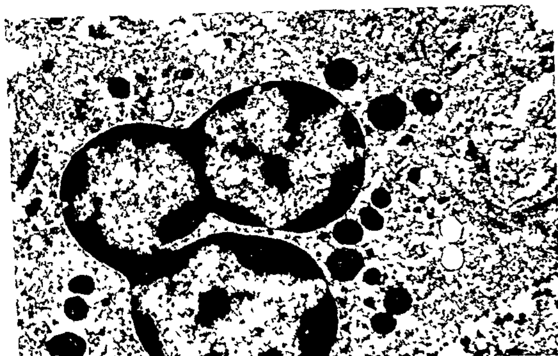


Fig 8 Tumour cell with large lobulated nucleus with chromatin condensation arround the nuclear membrane and electron dense dark round bodies without membrane limitations and some vesicles in the cytoplasma (7 800 ×)

epithelial elements were the cells of origin

In the present case, electron microscopy did not reveal any of the above mentioned features, this, however, might be due to improper fixation method The presence of two small adenomatous structures in the deeper parts of the present tumour is difficult to interprete, they did not show any direct contact with the tumour cells that could be interpreted as a transition between epithelial and apparently mesenchymal cells

I am indebted to Dr *T Winding* for access to records at the Assens Hospital

REFERENCES

1 *Andrea V C, Rattchev, R & Nikolova, D* Malignant Osteoclastoma of the Skin Brit J Derm *76* 40–44, 1964

2 *Dorney, P* Osteoclastoma of the Heart Brit J Heart *29* 276–278, 1967

3 *Fry, H J B* Osteoclastoma (Myeloid Sarcoma) of the Human Female Breast Journal of Path. and Bac. *30* 529–536, 1972

4 *Gonzales F & Karnovsky M* Electron Microoscopy of Osteoclasts in Healing Fractures of Rat Bone The journal of Biophysical and Biochemical Cytology *9* 299–316 1961

5 *Gonzales Licea, A Yardley, J H & Hartmann W H* Malignant tumours of the Breast with bone Formation (Studies by Light and Electron Microscopy) Cancer *20* 1234–1246, 1967

6 *Guccion J G & Enzinger, F M* Malignant Giant cell Tumours of Soft Parts Cancer *29* 1518–1529, 1972

7 *Hone A* An Electron Microscopic Observation of Giant Cells and Stromal Cells in the benign Giant Cell tumour of Bone. Fukuoka Acta Medica *52* 817 828, 1961

8 *Oberling C* Retroperitoneal Xanthogranuloma Amer J Cancer *23* 477–489 1935

9 *Rather, L J* Giant Cell Tumours of the Thyreoid Stanford Med Bulletin *8* 202–208, 1950

10 *Rosal, J* Carcinoma of Pancreas Simulating Giant Cell Tumour of the Bone Cancer *22* 333 344 1968

11 *Rosai Uribe, A, Ring A M & Rappaport, H* Metastazing Retroperitoneal Fibroxanthoma (Malignant Fibroxanthoma) Cancer *26* 827–831, 1970

Fig 6 Part of a giant cell with multinucleation and endoplasmatic reticulum (8,400 ×)

Fig 7 Some mononuclear tumour cells showing some endoplasmatic reticulum, small granules and fine threads in the cytoplasma The cell borders are surrounded by a zone of finely granular and moderately electron dense material (8,400 ×)

Acta path microbiol scand Section A *81*, 145–147, 1973

DELAYED HYPERSENSITIVITY AND HIGH BLOOD PRESSURE IN MAN

FINN OLSEN and BIRTE LOFT

Bispebjerg Hospital, Medical Department B
(Heads Mogens Bjorneboe, M D, Flemming Quaade, M D &
Aage Christian Thomsen, M D)

By means of the leucocyte migration technique it is demonstrated that a hypersensitivity of the delayed type seems to be involved in the hypertensive disease in patients suffering from primary essential hypertension The migration index is lower than 1 0 in the hypertensive patients, indicating an inhibition of the leucocyte migration, while the index in normotensive control persons shows practically no inhibition The hypersensitivity is directed *towards antigenic substances in the aortic wall of rabbits*

A hypersensitivity of the delayed type may be involved in some cases of hypertensive vascular disease in rats (*Olsen* 1971), and in histological sections from the kidneys and the periadrenal tissue of hypertensive patients it is possible to demonstrate an infiltration of mononuclear cells into the walls of arterioles (*Olsen* 1972 a) This cellular infiltration is possibly due to a hypersensitivity of the delayed type against antigenic substance(s) in the arterial walls

The aim of the present work has been to study whether a delayed type of hypersensitivity is involved in patients with primary essential arterial hypertension, using the leucocyte migration technique (*Bendixen & Søborg* 1969)

METHODS AND MATERIAL

On account of difficulties in obtaining aseptic arterial tissue from human subjects without cadaverosis, homogenized aortic tissue from rabbits has been used as antigenic substance in this study

Immediately after killing a healthy rabbit the thoracic and abdominal part of the aorta was removed and the aortic tissue homogenized The homogenized tissue was extracted in *Hank s* buffered salt solution during the night at a temperature of 4° C After centrifugation the supernatant was pipetted off from the sediment, and the concentration of the protein in the supernatant was measured Increasing concentrations of the aortic proteins were placed in the culture chambers and the migration area of the leucocytes of hypertensive patients and normotensive control persons was measured The results were given as the migration index which is defined as the ratio between the migration area of antigen containing and antigen-free culture

For a valuation of the specificity of the leucocyte migration technique in culture chambers containing aortic proteins, the migration index was measured in culture chambers containing equal concentrations of protein from homogenized and extracted tissue which was poor on vessels, namely the peritoneum of the rabbit

Six patients suffering from primary systemic hypertension were examined for a delayed hypersensitivity directed against antigenic substances in the aortic wall from rabbits The blood pressure of the patients was measured to 210–250 mm Hg systolic and 120–150 mm Hg diastolic The patients were examined for a secondary hypertension, but reno-

Received 2 x 72 Accepted 2 xi 72

Requests for reprints should be addressed to *Finn Olsen*, Bispebjerg Hospital, Medical Department B Bispebjerg Bakke 23, 2400 NV, Copenhagen Denmark

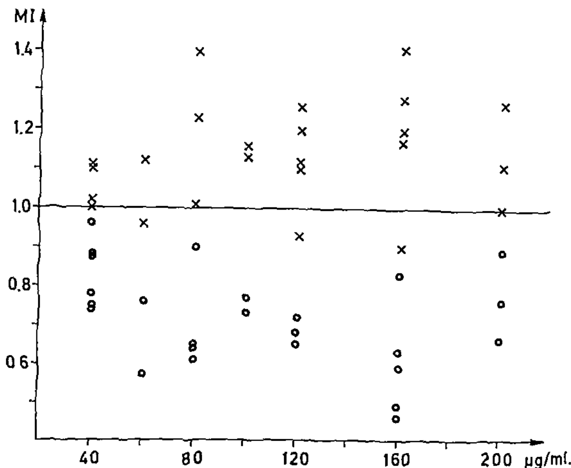


Fig 1 Hypertensive patients (o) Normotensive control persons (x) Abscissa Increasing concentrations of aortic proteins in the culture chambers Ordinate Migration index (MI) *The figure shows that the* migration index of all the hypertensive patients is lower than 1 0 The index decreases with increasing concentrations of the aortic proteins, reaching the lowest indices about 160 micrograms per ml of aortic proteins in the culture chambers Only three indices from the normal control persons are below 1 0, the lowest being 0 9 The rest of the indices is higher or equal to 1 0

vascular hypertension, pheochromocytoma, endocrinolic disease, and aortic coarctation were ruled out in all the cases The age of the patients, four men and two women, varied from 31 years to 62 years Two of the patients were being treated with *hydroflumethiazidum and methyldopum, while anti*-hypertensive treatment of the rest of the patients had not yet been instituted at the time of study of the leucocyte migration None of the patients had been immunized with blood transfusions, but the two women had been pregnant

The control persons were healthy normotensive persons at ages varying from 28 to 46 years The control persons comprised two women and three men

RESULTS

The results of the leucocyte migration given as the migration index are seen in Fig 1 This figure shows that the migration index from all the hypertensive patients is less than 1 0, and 76 per cent of the migration indices are lower than 0 8 The lowest indices are about 0 5 These results show an obvious inhibition of the leucocyte migration in the culture *chambers containing aortic antigenic substances*

The migration index from the normal per-sons shows that practically no inhibition of the leucocyte migration has taken place *in the*

culture chambers Only three indices are lower than 1 0, the lowest being 0 9, while the other 21 indices show values equal to or higher than 1 0, corresponding to about 88 per cent of the migration indices

In the cases in which a solution of protein from the peritoneum of the rabbit was placed in the culture chambers in stead of protein from the aortic wall, the migration indices from the hypertensive patients and the normotensive control persons were equal, varying from 0 93 to 1 09 These results show that the method used was specific

DISCUSSION

The leucocyte migration technique is an *in vitro* method for the demonstration of a hypersensitivity of the delayed type

In experimental studies in rats it has been demonstrated, using transfers of thoracic duct lymphocytes, that a hypersensitivity of the delayed type is involved in some cases of hypertensive vascular disease (*Olsen* 1971) Therefore, it has been interesting to examine whether a delayed type of hypersensitivity was involved in the hypertensive disease in man too The presented results show that such a hypersensitivity can be demonstrated in hypertensive patients The hypersensitivity is directed towards unknown antigenic substances in the vessel wall, but the role of such hypersensitivity in the pathogenesis of the arterial hypertension or the hypertensive vascular disease is unknown

In this study, antigenic substances from the aorta of a rabbit have been used As mentioned previously in this paper, the aorta from rabbits was used on account of the difficulties in obtaining aseptic tissue from humans

The study supports previous findings which indicate that a hypersensitivity of the delayed type may be involved in the hypertensive vascular disease (*Olsen* 1970, 1972 a) It is, however, only a preliminary study of the delayed type of hypersensitivity in the hypertensive vascular disease in man, and will be followed up by examinations in which antigenic substances from arteries of human subjects will be used

The fact that immunological factors are involved in the hypertensive disease has also been demonstrated by the finding of increased values of immunoglobulins in the serum from hypertensive patients (*Ebringer* & *Doyle* 1970, *Olsen* 1972 b), indicating a humoral type of hypersensitivity Furthermore, deposition of complement takes place in the damaged arterioles and glomeruli in cases of malignant hypertension (*Paronetto* 1965)

REFERENCES

1) *Bendixen, G* & *Søborg, M* A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man Danish medical Bulletin *16* 1–6, 1969
2) *Ebringer, A* & *Doyle, A E* Raised serum IgG levels in hypertensive vascular disease British med J *2* 146–149, 1970
3) *Olsen, F* Evidence for an immunological factor in the hypertensive vascular disease Acta path microbiol scand (A) 79 22 26, 1971
4) *Olsen, F* Inflammatory cellular reaction in hypertensive vascular disease in man Acta path microbiol scand (A) *80* 253 256, 1972 a.
5) *Olsen, F* Immunological factors and high blood pressure in man. Systemic hypertension and raised levels of immunoglobulins in the serum Acta path microbiol scand (A) *80* 257 259, 1972 b
6) *Paronetto, F* Immunocytochemical observations on the vascular necrosis and renal glomerular lesions of malignant nephrosclerosis. Amer J Path *46* 901 916 1965

Acta path microbiol scand Section A 81, 148–158, 1973

TESTIS WEIGHT AND THE HISTOLOGY OF THE PROSTATE IN ELDERLY MEN

An Analysis in an Autopsy Series

THORSTEIN B HARBITZ

Ullevål Hospital, Department of Pathology, University of Oslo Oslo, Norway
Head Kristen Arnesen, M D

As part of a study of the pituitary gland, adrenal glands and the testis in men with hyper plasia and neoplasia of the prostate, combined testis weight was analysed in a consecutive autopsy series of 172 men over 40 years of age None had clinically manifest carcinoma of the prostate or had previously been subjected to prostatic surgery Testis weight varied widely at all ages and showed no clear association with age Weights below 20 grams were shown in 21 patients (12 2 per cent) and were associated with wasting disease and low body weight No relationship between the testis weight *per se* and prostatic histology could be demonstrated at multiple regression analysis However, in patients who had benign nodular hyperplasia of the prostate, testis weight showed a *significant decrease with* increasing age Four of the 54 patients who had carcinoma of the prostate had testis weight under 20 grams Twenty four patients who had undergone prostatic surgery for benign prostatic hyperplasia showed testis weights which were similar to those in non operated cases

Testicular hormones are of major importance for the normal function and growth of the prostate (*Moore et al* 1930, *Huggins* 1947, *Price & Williams Ashman* 1961) Pathological growth of the human prostate is increasingly common after the age of 40 years (*Harbitz & Haugen* 1972) Clinical observations (*Huggins et al* 1941, *Moore* 1944), morphological studies of endocrine organs (*Sommers* 1956, 1957) and biochemical analyses of human plasma and urine (*Bulbrook et al* 1959, *Stern et al* 1964, *Weiner et al* 1966, *Isurugi* 1967) have suggested that complex disturbances in the function of endocrine glands are important factors in the pathogenesis of benign nodular hyperplasia and carcinoma of the prostate

Received 6 x 72 Accepted 6 x 72

Requests for reprints should be addressed to *Department of Pathology, Ullevål Hospital, Oslo 1*

The aim of the present investigation is to analyse testis weight in men with various forms of pathological growth of the prostate Correlations between changes in testis weight and serum levels of testicular and pituitary hormones were recently demonstrated in rats (*Amatayakul et al* 1971) Similarly, reduced testicular mass may reflect alterations in gonadal function in man (*Hudson et al* 1970) The relation between testis weight and prostatic histology may therefore give some indications about the role of the testis in the pathogenesis of hyperplastic and neoplastic disease of the prostate

In man, several factors may interfere with testis weight, and thus confound the analysis of its relation to pathological growth in the prostate Hence, the relations between testis weight and age, body weight, body length, cause of death, duration of terminal illness, steroid hormone treatment, diabetes mellitus

and liver cirrhosis have been studied. Thereafter, the relationship between testis weight and different forms of prostatic histology was analysed by multiple regression analysis, including a selected set of the more important confounding veriables.

This investigation forms part of a comprehensive autopsy study in elderly men, which has been performed to investigate the association between pathological growth of the prostate and morphological changes in the testes, the pituitary gland and the adrenal glands.

MATERIAL AND METHODS

The present material included 207 consecutive autopsies of male patients over 40 years of age who died at Ullevål Hospital during a 3 months period 1967–68. This material also formed the basis of previous studies of prostatic histology (*Harbitz* & *Haugen* 1972) and prostatic weight (*Haugen* & *Harbitz* 1972). Thus, the autopsy routine and the technique for examination of the prostate have been previously described.

Patients who had undergone prostatic surgery (24) or treatment with oestrogenic hormone (diethylstilboestrol) (4) or both (2) were analysed separately. Four patients manifesting secondary neoplastic involvement of the testes or prostate and one patient with seminoma of the testis were excluded from the present study. Thus 172 patients were eligible for the main analysis of testis weight.

Testis Weight

Testis weight is given as the combined weight of both testes before fixation and after the removal of the epididymis.

Histological Classification of the Prostate

Three to six whole sections through the prostate perpendicular to the urethra at 4–6 mm intervals were prepared for microscopical examination.

According to the procedure and histological criteria previously described (*Harbitz* & *Haugen* 1972), the presence of normal prostatic histology (N), benign nodular hyperplasia (BNH), carcinoma (C), atypical glandular proliferation (AGP) and diffuse atrophy (DA) was noted for each gland. The presence of atypical glandular proliferation was not specified in glands where carcinoma was diagnosed. The histological findings in the prostates of the 172 patients included in the main analysis are presented in Table 1.

Clinical Data

Clinical data were recorded from the clinical notes and prepared for computer analysis.

Statistical Analysis

Modified Student's t tests accounting for unequal variances and numbers of individuals were used for testing differences between arithmetic means and for testing differences between slopes of regression lines (*Snedecor* & *Cochran* 1967). n_A and n_B being the number of observations in the groups to be compared. p values were based on the least of $n_A - 1$ and $n_B - 1$ (for means) and $n_A - 2$ and $n_B - 2$ (for slopes) degrees of freedom. P values below 0.05 were regarded statistically significant.

Adjustment for age differences was performed according to the indirect method of standardization (*Armitage* 1971), using the age specific mean testis weights of the main material of 172 patients as standard weights.

Multiple regression analysis. Multiple regression analysis was applied as previously described (*Haugen* & *Harbitz* 1972), using testis weight (X_1) as the *dependent* variable. The following factors either bivariate (1 0, labelled X_2 to X_{12}) or continuous (labelled X_{13} to X_{15}) were treated as *explanatory* (independent) variables.

Histology of the Prostate

- X_2 Benign nodular hyperplasia (BNH)
- X_3 Atypical glandular proliferation (AGP)
- X_4 Carcinoma (C)
- X_5 Diffuse atrophy (DA)

Cause of Death

- X_6 Cardiovascular disease*
- X_7 Malignant tumour

Duration of Final Illness

- X_8 1–7 days
- X_9 >7 days

Other

- X_{10} Steroid hormone treatment**
- X_{11} Diabetes mellitus

---

* Includes death from myocardial infarction (49 cases), cerebrovascular and peripheral vascular disease (16+8 cases), rheumatic valvular disease (4 cases)), miscellaneous cardiovascular disorders (11 cases).

** Other than oestrogenic hormones. Includes treatment with corticosteroids (7 cases), anabolic steroids (nortestosterone) (5 cases) or both (11 cases).

TABLE 1 *Histology of the Prostate by Age in 172 Patients*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40-49	1	1	2	0	0	0	0
50-59	11	5	10	3	1	5	0
60-69	7	1	22	15	5	6	1
70-79	0	1	28	21	0	5	1
80-89	0	0	9	7	0	2	0
90 +	0	0	0	2	0	0	0
Total	19	8	71	48	6	18	2

N – normal histology, *DA* – diffuse atrophy, BNH – benign nodular hypertrophy C = carcinoma AGP = atypical glandular proliferation

X_{12} Liver cirrhosis
X_{13} Age
X_{14} Body weight
X_{15} Body lenght

Initially, forward stepwise regression analysis was run until all explanatory variables which were partially significant at the 5 per cent level at each step, were included Thereafter the selected variables together with all groups of prostatic histology (X_2 X_5), were included in the full multiple regression analysis Regression coefficients were calculated according to the method of least squares Differences between regression coefficients for the various groups of prostatic histology were tested by an F test (*Scheffe* 1959)

The analysis was based on a standard program for multiple regression (NRSR) developed at The Norwegian Computing Center Oslo and was conducted on a Univac 1108 computer

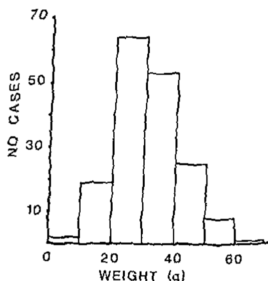


Fig 1 Distribution of testis weight at autopsy in the main series of 172 men aged 40 years or more

RESULTS

The frequency distribution of testis weights within the main set of 172 patients described above is presented in Figure 1 The distribution histogram was close to that of the normal distribution

The scatter of testis weight was wide at all ages, and no clear association with age as expressed by the correlation coefficient (r), could be demonstrated ($r = -0\ 115$ $p > 0\ 10$) (Fig 2 a) The standard deviations within 10-year age groups were high, and the mean weights in the sixth or seventh decade did not differ significantly from those in the higher decades (Table 2)

TABLE 2 *Testis Weight (Grams) in Men Previously not Subjected to Prostatic Surgery or Treatment with Oestrogenic Hormones*

Age	No patients	Testis weight Mean	SD
40–49	4	20 7	0 5
50–59	35	32 4	9 6
60–69	57	33 0	9 6
70–79	56	31 8	11 0
80–89	18	28 3	11 8
90 +	2	24 2	11 5
All	172	31 6	10 4

SD Standard deviation

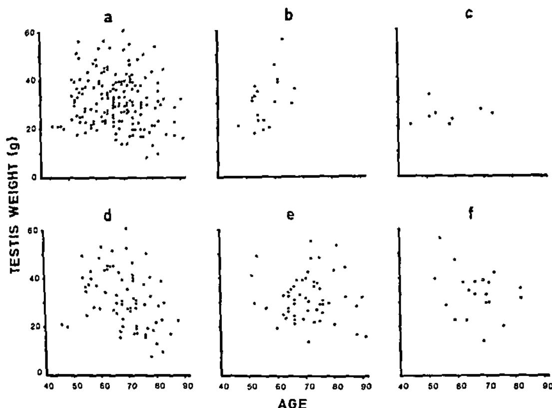


Fig 2 Testis weight plotted against age in the main series of 172 men over 40 years of age (a), and in subgroups of cases with normal histology (b), diffuse atrophy (c), benign nodular hyperplasia only (d), carcinoma with (●) or without (O) benign nodular hyperplasia (e), and atypical glandular proliferation with (●) or without (O) benign nodular hyperplasia (f) of the prostate

At all ages, the majority of patients had combined testis weights between 20 and 40 grams (Fig 2 a) Weights below 20 grams were seen in 21 patients (12 2 per cent), among whom 19 had protracted immobilization and wasting disease before death The relative frequency of such low weights increased with age, whereas the proportion of patients with high testis weights (more than 40 grams) remained fairly constant at all ages

The low testis weights observed in the four patients under 50 years of age could be due to long lasting wasting disease in two of the patients, whereas a plausible explanation for such low weights was not obvious in the remaining two patients in this age group No marked increase in testis weight from the fifth to the sixth decade has been observed in other studies (*Stieve* 1930, *Olesen* 1948) Hence, the gonadal weights in these patients are regarded as outliers and not representative of men in the fifth decade

Table 3 presents mean testis weights in relation to cause of death duration of final illness, treatment with steroid hormones other than oestrogens, diabetes mellitus and liver cirrhosis Mean testis weight in patients dying from malignant tumours was significantly lower than that in patients dying from cardiovascular disease ($p<0\ 005$) Patients with protracted duration of the terminal illness (more than 7 days) had significantly lower mean testis weight than those who died with in 1 day after onset of symptoms ($p<0\ 001$) Patients who died from malignant tumours and those dying after protracted disease had low body weights (56 7 and 59 4 kg) as com-

TABLE 3 *Testis Weight (Grams) and Cause of Death, Duration of Final Illness Steroid Hormone Treatment, Diabetes Mellitus, and Liver Cirrhosis*

	No patients	Testis weight		
		Observed mean	S D	Age adjusted mean
Cause of death				
Cardiovascular disease	88	33 9	10 3	33 8
Malignant tumour	44	27 6	9 5	27 6
Other causes	40	30 9	10 4	31 2
	172			
Duration of final illness				
<1 day	37	36 1	8 5	35 4
1–7 days	35	35 8	10 5	35 8
>7 days	100	28 5	9 9	28 7
	172			
Other				
Steroid hormone treatment*	23	28 3	8 5	28 3
Diabetes mellitus	8	30 9	8 4	31 9
Liver cirrhosis	5	28 0	15 8	27 2
All	172	31 6	10 4	

* Other than oestrogenic hormones
S D Standard deviation

pared with patients dying from cardiovascular disease or those dying within 1 day after onset of symptoms (67 1 and 71 2 kg) The low testis weights in patients with debilitating or protracted disease therefore seemed associated with their low body weights

Mean testis weight in men who had been treated with steroid hormones other than oestrogens was lower than that of the whole series (172 cases) (Table 3) Again this may be due to the fact that the majority of patients in this group died from malignant tumours

TABLE 4 *Mean Testis Weight (Grams) by Histology of the Prostate* and Age of Patients*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	(21 0)	(21 0)	20 5			–	–
50–59	26 8	25 4	37 2	39 3	(29 8)	38 8	–
60–69	39 6	(27 1)	35 1	29 8	27 6	29 9	(38 6)
70–79	–	(24 6)	30 8	33 5	–	31 9	(32 8)
80 +	–	–	22 6	32 1	–	33 4	
All	31 2	25 0	31 7	32 2	27 9	32 1	35 7
S D	10 0	4 0	11 8	9 1	5 5	9 4	4 1

* For abbreviations and number of patients see Table 1
Figures in brackets refer to single observations
S D Standard deviation

after protracted terminal illness Diabetics had mean testis weight which was close to the total mean Mean testis weight in patients with liver cirrhosis was low, but the standard deviation was high

Patients with diffuse prostatic atrophy generally had lower testis weight than patients with normal histology or pathological growth of the prostate (Table 4) Rather low testis weights were also observed in patients with prostatic carcinoma not accompanied by benign nodular hyperplasia Total mean testis weights in patients with prostatic hyperplasia, either alone or together with atypical glandular proliferations or carcinoma, were higher and similar to the mean in individuals with normal prostatic histology However, due to variation in the age distribution within the groups, it seems more meaningful to compare age specific than total means Among patients with normal histology of the prostate, testis weight increased significantly from the sixth to the seventh decade ($p<0\,02$), and among all patients 60–69 years of age, those with normal prostatic histology had the highest mean testis weight Age specific mean weights in subjects with prostatic hyperplasia alone steadily decreased with age from the sixth decade and onwards A similar tendency was not evident in the groups of benign nodular hyperplasia combined with either carcinoma or atypical glandular proliferation

Figures 2 b–2 f show plots of testis weight and age within groups of prostatic histology The scatter was generally wide at all ages in all groups, with the exception of those who had diffuse atrophy of the prostate In men with normal histology of the prostate, testis weight increased with age ($r = 0\,490$, $p < 0\,05$) A few cases of weights below 20 grams or over 40 grams were observed in all histological groups except the DA group A comparison of patients with benign hyperplasia only, carcinoma, atypical glandular proliferation and normal histology of the prostate, showed no obvious difference in the frequency distribution of testis weights

The relationship presented as regression lines between testis weight and age in patients with different histological diagnoses of the prostate is shown in Figure 3 As patients under 50 years of age were few and represented only in some of the histological groups, only patients aged 50 years and more were included The regression coefficient ($b = -0\,630$) in patients with prostatic hyperplasia alone (BNH) differed significantly from that ($b = -0\,122$) in patients carrying both prostatic carcinoma and benign hyperplasia (BNH+C) ($t = 2\,240$, $p < 0\,05$) Among the former, testis weight decreased gradually with age, the correlation being statistically highly significant ($r = 0\,446$, $p < 0\,001$) On the other hand, testis weight in patients with both prostatic carcinoma and benign nodular hyperplasia declined only slightly with advancing age, and the correlation coefficient ($r = -0\,111$) did not differ significantly from zero ($p>0\,10$) The regression line for testis weight on age in patients with benign nodular hyperplasia and atypical glandular proliferation (BNH + AGP) was between the lines for the BNH and BNH + C groups, and did not differ significantly from either the slopes of these two lines nor from zero ($r = -0\,307$, $p > 0\,10$) Testis weight increased with age in men over 50 years of age with normal prosta-

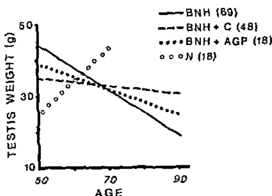


Fig 3 Regression of testis weight on age in men more than 50 years of age with benign nodular hyperplasia only (BNH) benign nodular hyperplasia and carcinoma (BNH+C), benign nodular hyperplasia and atypical glandular proliferation (BNH+AGP), and normal histology (N) of the prostate Figures in brackets indicate number of cases in each group

TABLE 5 *Testis Weight (Grams) in Patients Previously Subjected to Prostatic Surgery**

Age	No patients	Testis weight Mean	S D
50–59	3	27 6	4 3
60–69	5	30 6	10 7
70–79	7	27 6	9 0
80–89	8	30 7	10 2
90 +	1	(26 5)	–
All	24	29 2	8 8

* Two patients subjected to both prostatectomy and treatment with oestrogenic hormones not included

S D Standard deviation

tic histology ($r = 0\,438$, $0\,05 < p < 0\,10$), but the regression line cannot be directly compared to those of the other histological groups because of the differences in age distribution

Testis weight in patients who had been subjected to prostatic surgery (transvesical prostatectomy or transurethral resection) appears from Table 5 The age specific mean weights did not differ significantly from those in non operated cases (Table 2)

Oestrogenic hormones had been given to six patients for periods varying from one to 50 months before death Their testis weights showed a wide range (1 9–22 2 grams), and the mean was considerably lower (14 0 grams) than that of patients who had no oestrogen treatment

Simple correlation analyses were performed in the main series prior to the multiple regression analysis as a preparatory procedure, and the relationship, expressed by correlation coefficients, between testis weight and each of the explanatory variables to be included in the multiple regression analysis (X_2–X_{15})

TABLE 6 *Relationship between Testis Weight and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variable	X_1 *Testis weight ($n_1 = 172$)* Correlation coefficient	Significant at level
*Histology of the prostate**		
X_2 BNH (n – 137)	0 104	0 174
X_3 AGP (n – 20)	0 068	0 376
X_4 C (n = 54)	0 020	0 796
X_5 DA (n – 8)	–0 142	0 063
Cause of death		
X_6 Cardiovascular disease (n – 88)	0 236	0 00?
X_7 Malignant tumour (n = 44)	–0 226	0 003
Duration of final illness		
X_8 1 7 days (n = 35)	0 206	0 007
X_9 >7 days (n – 100)	–0 356	<0 001
Other		
X_{10} Steroid hormone treatment§ (n = 23)	–0 125	0 103
X_{11} Diabetes mellitus (n – 8)	–0 016	0 835
X_{12} Liver cirrhosis (n – 5)	–0 060	0 431
X_{13} Age (n – 172)	–0 115	0 133
X_{14} Body weight (n = 172)	0 353	<0 001
X_{15} Body length (n – 172)	0 109	0 155

* For abbreviations see Table 1

(for bivariate variables) or

§ Other than oestrogenic hormones

appears from Table 6 Testis weight was positively correlated with body weight, death from cardiovascular disease and short duration of the final illness (1–7 days), and negatively correlated with malignant tumour as a cause of death and protracted illness before death (> 7 days), all at high significance levels Obviously, these are not truly independent variables *Death from cardiovascular* disease is frequently associated with short duration of final illness and high body weight, whereas death from malignant tumours is associated with long duration of final illness and low body weight

Testis weight showed no correlation with any histological pattern of the prostate at the chosen level for statistical significance

Multiple Regression Analysis

At the stepwise regression analysis, only final illness of more than 7 days' duration (X_9) and body weight (X_{14}) reduced the total variance of testis weight significantly, and both variables were selected at high significance levels ($p \approx 0\ 001$) However the multiple correlation coefficient (R) for the two was low (0 429), and the proportion of the variance of testis weight that could be attributed to its linear regression on final illness of long duration and body weight (expressed as R^2) was only 18 4 per cent The relationship between testis weight and the selected regressors can be predicted from the regression equation Testis weight *in the individual case in* this series would increase by 0 189 grams per kilogram body weight, whereas duration of the final illness more than 7 days on average caused a reduction of testis weight by about 5 5 grams

At full regression analysis including the regressors for prostatic histology (X_2–X_5) and the two regressors selected at stepwise procedure (Table 7), testis weight showed no convincing relationship to any group of prostatic histology Neither did the regression coefficients of the various histological groups differ significantly from each other ($F \approx 0\ 866$, $f_1 = 4$, $f_2 = 165$, $p > 0\ 25$) The explanatory value of the multiple correlation coefficient increased only from 18 4 to 20 1 *per cent* by the introduction of prostatic histology in the regression Thus, if duration of final illness and body weight are accounted for, prostatic histology as classified here shows practically no association with testis weight as such

Separate stepwise regression analyses were performed in the case of the 69 patients with benign nodular hyperplasia only, the 48 patients with both carcinoma and benign hyper plasia the 18 patients with both atypical glandular proliferation and benign hyper-

TABLE 7 *Testis Weight and Histology of the Prostate Full Regression Analysis*

Explanatory variable	X_1 *Testis weight* ($n_1 = 172$)		
	Partial correlation coefficient	Partial regression coefficient	Significant at level
*Histology of the prostate**			
X_2 BNH (n 137)	0 089	2 336	0 251
X_3 AGP (n = 20)	0 068	2 063	0 381
X_4 C (n = 54)	0 066	1 420	0 395
X_5 DA (n 8)	0 005	0 244	0 951
Other			
X_9 >7 days (n = 100)	−0 259	5 549	0 001
X_{14} Body weight (n = 172)	0 258	0 192	0 001
Multiple correlation coefficient (R)	0 448		<0 001

* For abbreviations see Table 1 n and n_1 For explanation see Table 6

TABLE 5 *Testis Weight (Grams) in Patients Previously Subjected to Prostatic Surgery**

Age	No patients	Testis weight	
		Mean	S D
50–59	3	27 6	4 3
60–69	5	30 6	10 7
70–79	7	27 6	9 0
80 89	8	30 7	10 2
90 +	1	(26 5)	–
All	24	29 2	8 8

* Two patients subjected to both prostatectomy and treatment with oestrogenic hormones not included

S D Standard deviation

tic histology ($r = 0.438$, $0.05 < p < 0.10$), but the regression line cannot be directly compared to those of the other histological groups because of the differences in age distribution

Testis weight in patients who had been subjected to prostatic surgery (transvesical prostatectomy or transurethral resection) appears from Table 5 The age-specific mean weights did not differ significantly from those in non operated cases (Table 2)

Oestrogenic hormones had been given to six patients for periods varying from one to 50 months before death Their testis weights showed a wide range (1 9–22 2 grams), and the mean was considerably lower (14 0 grams) than that of patients who had no oestrogen treatment

Simple correlation analyses were performed in the main series prior to the multiple regression analysis as a preparatory procedure, and the relationship, expressed by correlation coefficients, between testis weight and each of the explanatory variables to be included in the multiple regression analysis (X_2–X_{15})

TABLE 6 *Relationship between Testis Weight and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variable	X_1 Testis weight (n_1 – 172) Correlation coefficient	Significant at level
*Histology of the prostate**		
X_2 BNH (n – 137)	0 104	0 174
X_3 AGP (n – 20)	0 068	0 376
X_4 C (n = 54)	0 020	0 796
X_5 DA (n – 8)	–0 142	0 063
Cause of death		
X_6 Cardiovascular disease (n – 88)	0 236	0 002
X_7 Malignant tumour (n – 44)	0 226	0 003
Duration of final illness		
X_8 1 7 days (n = 35)	0 206	0 007
X_9 >7 days (n = 100)	0 356	<0 001
Other		
X_{10} Steroid hormone treatment§ (n = 23)	–0 125	0 103
X_{11} Diabetes mellitus (n – 8)	–0 016	0 835
X_{12} Liver cirrhosis (n – 5)	0 060	0 431
X_{13} Age (n = 172)	–0 115	*0 133*
X_{14} Body weight (n – 172)	0 353	<0 001
X_{15} Body length (n – 172)	0 109	0 155

* For abbreviations see Table 1

n_1 Number of cases in which testis weight was recorded

n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continuous variables)

§ Other than oestrogenic hormones

appears from Table 6 Testis weight was positively correlated with body weight, death from cardiovascular disease and short duration of the final illness (1–7 days), and negatively correlated with malignant tumour as a cause of death and protracted illness before death (> 7 days), all at high significance levels Obviously, these are not truly independent variables Death from cardiovascular disease is frequently associated with short duration of final illness and high body weight, whereas death from malignant tumours is associated with long duration of final illness and low body weight

Testis weight showed no correlation with any histological pattern of the prostate at the chosen level for statistical significance

Multiple Regression Analysis

At the stepwise regression analysis, only final illness of more than 7 days' duration (X_8) and body weight (X_{14}) reduced the total variance of testis weight significantly, and both variables were selected at high significance levels ($p = 0\ 001$) However, the multiple correlation coefficient (R) for the two was low (0 429), and the proportion of the variance of testis weight that could be attributed to its linear regression on final illness of long duration and body weight (expressed as R^2) was only 18 4 per cent The relationship between testis weight and the selected regressors can be predicted from the regression equation Testis weight in the individual case in this series would increase by 0 189 grams per kilogram body weight, whereas duration of the final illness more than 7 days on average caused a reduction of testis weight by about 5 5 grams

At full regression analysis including the regressors for prostatic histology (X_2–X_5) and the two regressors selected at stepwise procedure (Table 7), testis weight showed no convincing relationship to any group of prostatic histology Neither did the regression coefficients of the various histological groups differ significantly from each other ($F = 0\ 866$, $f_1 = 4$, $f_2 = 165$, $p > 0\ 25$) The explanatory value of the multiple correlation coefficient increased only from 18 4 to 20 1 per cent by the introduction of prostatic histology in the regression Thus, if duration of final illness and body weight are accounted for, prostatic histology as classified here shows practically no association with testis weight as such

Separate stepwise regression analyses were performed in the case of the 69 patients with benign nodular hyperplasia only, the 48 patients with both carcinoma and benign hyperplasia, the 18 patients with both atypical glandular proliferation and benign hyper-

TABLE 7 *Testis Weight and Histology of the Prostate Full Regression Analysis*

Explanatory variable	Y_1 *Testis weight* ($n_1 = 172$)		
	Partial correlation coefficient	Partial regression coefficient	Significant at level
*Histology of the prostate**			
X_2 BNH (n = 137)	0 089	2 336	0 251
X_3 AGP (n = 20)	0 068	2 063	0 381
X_4 C (n = 54)	0 066	1 420	0 395
X_5 DA (n = 8)	0 005	0 244	0 951
Other			
X_8 >7 days (n = 100)	−0 259	−5 549	0 001
X_{14} Body weight (n = 172)	0 258	0 192	0 001
Multiple correlation coefficient (R)	0 448		<0 001

* For abbreviations see Table 1   n and n_1 For explanation, see Table 6

plasia, and the 18 patients with normal histology of the prostate over 50 years of age At this procedure, the association between testis weight and age in men with benign hyperplasia observed at simple analysis (Fig 3) was confirmed Age was selected prior to body weight and at a highly significant level ($p < 0.001$) No significant association with age was detected within the other groups of prostatic histology

COMMENT

The interpretation of the results from this investigation should not be based upon the multiple regression analysis alone The findings at simple analysis should also be taken into account, especially if the explanatory value of the model is low

A large proportion of the patients in the present series of consecutive autopsies had protracted disease and malnutrition before death, and testis weights were lower than in previous autopsy series reported from Europe and U S A (*Stieve* 1930, *Olesen* 1948, *Sokal* 1964, *Calloway et al* 1965) However, the weights were similar to those in *Krieger's* (1921) series, in which the majority of patients suffered from chronic or debilitating disease These conditions are accompanied by reduction in testis weight (*Jackson* 1925 *Oiye* 1928, *Mulinos & Pomerantz* 1940), and a negative and significant correlation between testis weight and protracted illness was shown in the present study Hence, differences in the selection of the autopsy population may be responsible for the variation in testis weight observed in the various materials

A positive and statistically significant correlation between testis weight and body weight was demonstrated at single variable analysis and confirmed at the multiple regression analysis The fact that body length was not significantly correlated with testis weight indicates that testis weight depends rather upon nutritional state than upon body size as such

The associations between testis weight and death from cardiovascular disease, death from malignant tumour, and treatment with steroid hormones other than oestrogens observed at single variable analysis were not confirmed at multiple regression It is likely, therefore, that these associations are only due to intercorrelations of these factors and correlation with body weight and protracted terminal illness

Diabetes mellitus in men is commonly accompanied by sexual disorders due to hypogonadotrophic hypogonadism (*Schöffling et al* 1963) In the present series, a significant correlation between testis weight and diabetes mellitus could not be demonstrated However, the number of diabetics was small and the metabolic disease mostly of mild type

Atrophy of the testes is a common finding in male cirrhotics (*Bennett et al* 1950, *Robson* 1966), and disturbances in the oestrogen and androgen metabolism in these patients are well documented (*Glass et al* 1940 *Coppage & Cooner* 1965, *Korenmann et al* 1969) Testis weights in the few patients with liver cirrhosis in the present series varied widely, and no significant correlation between testis weight and the presence of liver cirrhosis was demonstrated

For reasons of analysis, the histological patterns of prostatic growth were included in the multiple regression analysis as explanatory variables and testis weight as the dependent variable The difference among the histological patterns of the prostate in their associations with testis weight was measured by the comparison of their regression coefficients No significant difference between them was detected, which means that testis weight *by itself* does not discriminate between various histological patterns of the prostate The reduced testis weight in patients with histologically diffuse atrophy of the prostate is probably related to the low body weight and long duration of the terminal illness in these patients

A significant correlation between testis weight and age was not demonstrable in the total material, confirming previous reports based on simple analysis (*Oiye* 1928, *Stieve* 1930, *Olesen* 1948, *Tillinger* 1957, *Sokal*

1964, *Calloway et al* 1965) On the other hand, classification of the patients according to prostatic histology revealed a negative and statistically significant correlation between testis weight and age in men with benign nodular hyperplasia alone The significance of this observation is uncertain Since the mean weight of the prostates with benign nodular hyperplasia increased with age, however (*Haugen* & *Harbitz* 1972), it may indicate that the size of the hyperplastic lesions is maintained or possibly even increases in spite of a reduction in testis weight Histological studies of the testes may give further information about the implications as regards the hormonal activity

As reflected by mean testis weight in this series, surgical treatment of benign prostatic hyperplasia, which involves removal of pro static tissue, did not affect the size of the testis. This probably implies that the endocrine functions of the testes are generally maintained after either transvesical or transurethral prostatectomy

Testicular atrophy subsequent to oestrogen treatment in patients with prostatic carcinoma is well known and was confirmed in the present studies of testis weight The reduction in size reflects marked disturbances in the endocrine function of the testis (*Robinson* & *Thomas* 1971), justifying that these patients were not included in the main analysis of testis weight in relation to prostatic histology

Reduced testis weight may be accompanied by disturbed gonadal function (*Hudson et al* 1970, *Amatayakul et al* 1971) Only few reports on the occurrence of prostatic carcinoma in men with hypogonadal states are available (for references, see *Arduino* 1967) Among the 172 patients in the present series who had not undergone prostatic surgery, clinically unsuspected prostatic carcinoma was found in four men with testis weight less than 20 grams The carcinomas were small (*Harbitz* & *Haugen* 1972), showed only limited local invasion, and are not likely to have caused occlusion of the distal spermatic tract with subsequent epididymo orchitis and testicular atrophy Although two of the patients had low body weight and an other had protracted disease before death, it is possible that the malignancy really developed in persons with subnormal size of the testes and disturbed gonadal function

The testis is the main source of both androgenic and oestrogenic hormones in men (*Baulieu* & *Robel* 1970, *Leach et al* 1956) In comparison with the hormone sensitive tissues of the testis, its hormone producing tissues make up a relatively small proportion of the gonadal mass Studies of the quantitative morphology of the testes in the present series are in progress to throw further light upon the pathogenesis of prostatic hyperplasia and neoplasia

---

I am indebted to Professors *Helge Stalsberg*, M D and *Knut Westlund*, M D, Institute of Medical Biology, *University of Tromso, Norway*, for helpful advice

Part of this study was performed while the author was a research fellow under *The Norwegian Society for Fighting Cancer* and *The University of Oslo* Financial support was thankfully received from *Grosserer N 4 Stangs Legat til Kreftsykdommers Bekjempelse*

REFERENCES

1 *Amatayakul, K, Ryan, R, Uozumi, T* & *Albert, A* A reinvestigation of testicular anterior pituitary relationships in the rat I Effects of castration and chryptorchidism. Endocrinology *88* 872–880, 1971

2 *Arduino, L J* Carcinoma of the prostate in sex chromatin positive (XXXY) Klinefelter's syndrome J Urol. *98* 234–240, 1967

3 *Armitage, P* Statistical methods in medical research Blackwell Scientific Publications, Oxford and Edinburgh 1971 504 pp

4 *Baulieu, E E* & *Robel, P* Catabolism of testosterone and androstenedione In Eik Nes, K B (Ed) The Androgens of the Testis Dekker, New York 1970 p 49–71

5 *Bennett, H S, Baggenstoss A H* & *Butt, H R* The testis breast and prostate of men who die of cirrhosis of the liver Amer J clin Path *20* 814–828, 1950

6 *Bulbrook, R D, Franks, L M* & *Greenwood, F C* Hormone excretion in prostatic cancer

An attempt to correlate urinary hormone excretion and clinical state Brit J Cancer *13* 45–58, 1959

7 *Calloway, N O, Foley, C F & Lagerbloom, P* Uncertainties in geriatric data II Organ size J Amer Geriatrics Soc *13* 20–28, 1965

8 *Coppage, W S, Jr & Cooner, A E* Testosterone in human plasma New Engl J Med *273* 902–907, 1965

9 *Glass, S J, Edmondson, H. A & Soll, S N* Sex hormone changes associated with liver disease Endocrinology *27* 749–752, 1940

10 *Harbitz, T B & Haugen, O A* Histology of the prostate in elderly men A study in an autopsy series Acta path microbiol scand Section A *80* 756–768, 1972

11 *Haugen, O A & Harbitz, T B* Prostatic weight in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *80* 769–777, 1972

12 *Hudson, B, Burger, H G, de Kretser, D M, Coghlan, J P & Taft, H P* Testosterone plasma levels in normal and pathological conditions In Rosemberg, E & Paulsen, C A (Eds) The Human Testis Advanc Exp Med Biol *10* 423–437, 1970

13 *Huggins, C* The prostatic secretion Harvey Lect *42* 148–193, 1947

14 *Huggins, C, Stevens, R E, Jr & Hodges, C V* Studies on prostatic cancer II The effects of castration on advanced carcinoma of the prostate gland Arch Surg *43* 209–223, 1941

15 *Isurugi, K* Plasma testosterone production rates in patients with prostatic cancer and benign prostatic hypertrophy J Urol *97* 903 908, 1967

16 *Jackson, C M* The effects of inanition and malnutrition upon growth and structure Churchill London 1925 p 403–416

17 *Korenmann, S G, Perrin, L E & McCallum, T* Estradiol in human plasma Demonstration of elevated levels in gynecomastia and in cirrhosis (Abstr no 144) J clin Invest *48* 45 a 1969

18 *Krieger, M* Über die Atrophie der menschlichen Organe bei Inanition Z angew Anat Konstitutionslehre *7* 87–134, 1921

19 *Leach, R B, Maddock, W O, Tokuyama, I & Paulsen, C A* Clinical studies of testicular hormone production Recent Progr Hormone Res *12* 377–403, 1956

20 *Moore, C R, Price, D & Gallagher, T F* Rat prostate cytology as a testis hormone indicator and the prevention of castration changes by testis extract injections Amer J Anat *45* 71–107, 1930

21 *Moore, R A* Benign hypertrophy and carcinoma of the prostate Occurrence and experimental production in animals Surgery *16* 152 167, 1944

22 *Mulinos, M G & Pomerantz, L* Pseudo-hypophysectomy A condition resembling hypophysectomy produced by malnutrition J Nutr *19* 493–504, 1940

23 *Oiye, T* Statistische und histologische Hodenstudien Mitt allg Path *4* 425–492, 1928

24 *Olesen, H* Morfologiske sperma og testis undersogelser Munksgaard, Kobenhavn 1948. 269 pp

25 *Price, D & Williams Ashmann, H G* The accessory reproductive glands of mammals In Young, W C (Ed) Sex and Internal Secretions, 3 ed vol I Williams & Wilkins Co, Baltimore 1961 p 366–448

26 *Robson, M C* Cirrhosis and prostatic neoplasms Geriatrics *21* 150–154 1966 (Dec.)

27 *Robinson, M R G & Thomas, B S* Effect of hormone therapy on plasma testosterone levels in prostatic carcinoma Brit med J *4* 391–394, 1971

28 *Scheffé, H The analysis of variance* John Wiley & Sons, Inc, New York 1959 p 25–51

29 *Schoffling, K, Federlin, M D Ditschuneit, H & Pfeiffer, E F* Disorders of sexual function in male diabetics Diabetes *12* 519–527, 1963

30 *Snedecor, G W & Cochran, W G* Statistical methods 6 ed The Iowa State University Press, Ames, Iowa 1967 pp 593

31 *Sokal, Z* Morphology of the human testes in various periods of life Folia morph *23* 102–111 1964

32 *Sommers, S C* Testicular spermatogenic cell *hypertrophy accompanying prostatic hyper*trophy and cancer Amer J Path *32* 185–199 1956

33 *Sommers, S C* Endocrine changes with prostatic carcinoma Cancer *10* 345 358 1957

34 *Stern, E Hopkins, C E Weiner J M & Marmorston, J* Hormone excretion patterns in breast and prostate cancer are abnormal Science *145* 716–719, 1964

35 *Stieve, H* Mannliche Genitalorgane In Mollendorff W von Handbuch der mikroskopischen Anatomie des Menschen Bd 7/II Springer, Berlin 1930 p 1–399

36 *Tillinger, K G* Testicular morphology A histopathological study with special reference to biopsy findings in hypogonadism with mainly endocrine disorders and in gynecomastia. *Acta endocr Suppl 30 13 38, 1957*

37 *Weiner, J M, Marmorston, J Stern F & Hopkins, C E* Urinary hormone metabolites in cancer and benign hyperplasia of the prostate A multivariate statistical analysis Ann N Y Acad Sci *125* 974–983 1966

cta path microbiol. scand Section A 81, 159 166, 1973

ENZYME HISTOCHEMICAL STUDIES OF THE LUNG OF DOMESTIC FOWL

The Effects of Unilateral Pulmonary Artery Occlusion

ESKO LANSIMIES and KARI KARKOLA

Cardio-Respiratory Research Unit and Department of Forensic Medicine
University of Turku, Finland

The enzyme histochemistry was studied in the lung of domestic fowl in normal state and after left pulmonary artery ligation (PAL) Immediately after PAL, non specific esterase activity increased in the epithelial cells and the tertiary bronchi Naphthylamidase reaction increased intensely in the epithelium and adjacent structures after PAL After PAL, air/blood capillary network showed pronounced tetrazolium reductase activity in a corona like circle around bronchi. It was concluded that the active transport mechanisms seem to be disturbed immediately after PAL but no severe ischaemic changes were observed A temporary activation of energy yielding process was noted in the capillary network near the tertiary bronchi A proliferative or inflammatory process is perceptible in the epithelium of the tertiary bronchi and the walls of the bronchial atria.

The avian lung and circulation have evolved from the reptilian system, but are functionally similar to those of mammals (7)

Avian lungs are suitable for histological and histochemical studies because they are rich in connective tissue and of a spongy consistence They do not collapse

The highly inadequate present knowledge of the circulation of the domestic fowl in general and circulation through lungs especially has been acknowledged quite recently (1) The dual circulation to the lungs as well as the response of bronchial circulation to pulmonary artery occlusion have been documented in several mammals including man (20 21) The cardiovascular effects of pulmonary artery occlusion in the domestic fowl have been mentioned only briefly (5) Preliminary reports have been published by us (14, 15)

The information obtained through routine histological examination of pulmonary changes will be published in detail later This study was conducted with a view to evaluate whether enzyme histochemical methods would improve the understanding of metabolic changes in the lung after ligation of the left pulmonary artery (PAL)

Received 10 viii 72 Accepted 31 x 72

Request for reprints should be addressed to *Esko Lansimies* Cardio-Respiratory Research Unit University of Turku SF 20520 Turku 52, Finland

MATERIAL AND METHODS

A total of 28 cockerels, bred by a professional farmer, served as experimental animals The first procedures were executed when they were two months old and weighed 400–700 g A left sided thoracotomy was done using local anaesthesia and the previously described method with minor modifications (5) The left pulmonary artery was clamped with neurosurgical clips The operational procedure was clean, but not sterile The animals were divided into groups of four animals, three of which were operated on while one non-operated

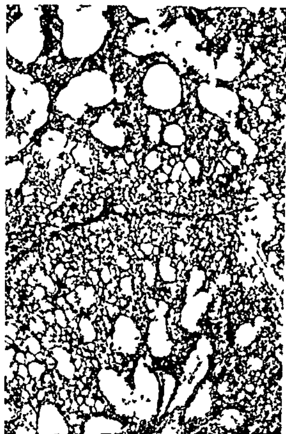


Fig 1 Haematoxylin eosin normal lung 90× Air/blood capillary network with div d ng septum

served as control At intervals of 2 hrs 1 2 4 10 days 1 month and 6 months the respective groups of animals were killed by decapitation and samples of both lungs were immediately removed for h sto logical and enzyme histochemical preparations PAL was ascertained in all cases

The preparative procedures were as follows

1 The sample was fixed in neutral formalin embedded in paraffin and stained for histological investigation by haematoxylin eosin (HE)

2 The sample was frozen immediately in iso pentane and maintained at 70° C in a mixture of acetone and dry ice Sections of 16 μm in th ck ness were cut in a cryostat rotary microtome and permitted to dry on a slide The following enzyme histochemical stainings were carried out alkaline phosphatase (10 13) nonspecific esterase (18) modifiec
10 min
midase
reductase (succinate dehydrogenase) (16) NADH and NADPH tetrazolium reductase (DPN and TPN diaphorase) (2) The name naphthylamidase is here employed to describe the activity of a group of enzymes commonly called aminopeptidases

RESULTS

Histological findings

HE – staining shows the histological structure of primary and secondary bronchi with high epithelium Tertiary bronchi (parabronchi) and the small pockets (atria) around the lat ter are lined by single cuboidal epithelium Tertiary bronchi are encircled by bronchial muscles with openings to the atria (1) Each tertiary bronchus is surrounded by at network of air and blood capillaries A connective tis sue septum, where the blood vessles run separates the tertiary bronchi into hexagonal lobuli as lung units (Fig 1)

At early phases after ligation of the left pulmonary artery, the lung appeared oede matous and some thrombosis was observed The structure was otherwise normal (Fig 2) Later on, the air capillaries were viable but blood capillaries probably came from arteries



Fig 2 HE stain ng two days after PAL 90× Oedema and thrombosis is seen



Fig 3 HE staining, 33 days after PAL 90× Thickening of the septum and atrophy of capillary network is clearly seen Note the vessels in the capillary network

newly formed in the air/blood capillary zone The connective tissue septa grew thicker and some degeneration could be observed (Fig 3)

Enzyme Histochemical Findings in the Control Group

Alkaline phosphatase activity is seen as black deposits in the endothelium, in the adventitia of blood vessels, and in connective tissue septa. Around the tertiary bronchi and atria, the activity is seen in the basal membranes of the epithelium Bronchial muscles and air capillary network stain slightly (Fig 4)

Non-specific esterase reaction is highly selective and shows distinctly the cuboidal epithelium of the tertiary bronchi and atria as well as the high epithelium of primary and secondary bronchi. Bronchial muscles are moderately active while all other components are inactive

Naphthylamidase reaction is scarce and nothing but occasional and slight activity is seen in some of the interatrial septa and the epithelium (Fig 5).

Succinate tetrazolium reductase and NADH- and NADPH-tetrazolium reductase show intense activity in all epithelial cells, moderate activity in the smooth muscles of bronchi and media of arteries Air/blood capillary network stain only very slightly (Fig 6)



Fig 4 Alkaline phosphatase, normal lung 100× The lower left corner shows the structure of a tertiary bronchus with atria A cross section of a pulmonary artery branch is presented in the upper right corner



Fig 5 Naphthylamidase normal lung 100× Only slight staining of the epithelium

Enzyme Histochemical Findings after Ligation

After two hours alkaline phosphatase showed decrease of activity in all active components except the endothelium of arteries The same phenomenon could be observed in the operated and non operated lung For 24 hours the situation remained unchanged but after two days the activity of the right lung recurred (Fig 7) After ten days the activity of the left operated lung was more intense than normal and showed staining especially in the blood capillary network close to the tertiary bronchi After 30 days the picture was similar to that seen after ten days (Fig 8) Six months later the structure suggested atrophy and the activity was less intense than that in control cases (Fig 9)

Non specific esterase activity was well preserved in the epithelium at all times After ten days, active cells in the air/blood capillary network appeared at a new site close to the tertiary bronchi This activity disappeared within 30 days Later on, slightly active cells were seen in connective tissue septa After six months the amount of epithelial cells seemed to decrease (Fig 10)

Naphthylamidase reaction was normal until day four, at which time activity in the epithelium began to increase The activity was very intense at ten days, but subsequently it decreased to normal level (Fig 11)

All three tetrazolium reductases showed the same distribution of activity at all times At four days, the activity of the epithelium was slightly decreased and almost inactive zones were formed beneath the septa At ten days and 30 days there was slight activity in the epithelium, but pronounced staining in small



Fig 6 NADPH tetrazolium reductase normal lung 100× Normal distribution of activity in the epithelium Moderate staining of the bronchial alveoli

cell groups forming a corona around the tertiary bronchi in blood capillary network In six months, this phenomenon disappeared and moderate activity of the epithelium remained (Fig 12)

DISCUSSION

So far, the enzyme histochemistry of the lung tissue has been studied in mammals only The cellularity of pulmonary tissue is very scarce and hence, some supporting medium by which to fill the alveoli is recommended (3) The structure of the avian lung is spongy and frozen sections can be cut without filling the bronchial tree

The main structure of connective tissue septa and blood vessels is very clearly demonstrated by the reaction of alkaline phosphata-



Fig 7 Alkaline phosphatase, 2 hours after occlusion 100× Diffusion and slight diminution of the activity



Fig 8 Alkaline phosphatase, 30 days after occlusion, 100× Increase of activity in the capillary network The rounded shape in the connective tissue septum is probably an enlarged bronchial artery

se No nuclear staining is noted out, if in evidence, it is considered an artifact (18) The real enzymatic activity of collagen fibres is questionable, but alkaline phosphatase activity of the fibres in proliferative processes has been reported (8, 11) According to ultrastructural investigations, the activity has been found to be localized to cellular membranes (9) In the rat lung alkaline phosphatase has been reported to occur in alveolar cells and

be seen in

at (3)

), non specific esterase seems to be highly selective in the fowl lung It stains only the epithelial cells of the tertiary bronchi and, even more pronounced, the high epithelium of the larger bronchi High activity has been observed in mammals (4) al-

11*



Fig 9 Alkaline phosphatase 6 months after occlusion 100× Activity has decreased and the structures seem to be atrophic but otherwise normal

though activity in the rat lung has been noticed only in the alveolar macrophages the colour in the epithelium is assumed to be an artifact (3) The cells remained active after unilateral pulmonary occlusion thus indicating that no remarkable damage had occurred The activity outside the ordinary sites may denote an appearance of histiocytes (12) around the tertiary bronchi The same explanation is valid as regards the pulmonary septa where slight activity was demonstrable The activity of non specific esterase is of great value whenever the condition of the epithelial cells of tertiary bronchi is to be estimated

The activity of naphthylamidase reaction in chickens is very scarce under normal circumstances A few days after the occlusion of the pulmonary artery there was a marked increase in activity in the epithelium of tertiary bronchi and in their vicinity According to various investigations proliferating connective tissue is known to be highly active (4) Inflammatory reaction is also connected with high activity (19) but the exact site of activity cannot be established because of the diffuse extracellular activity (17)

The activity of succinate tetrazolium reductase may indicate succinate dehydrogenase which is a mitochondrial enzyme. The other reductases studied are considered to be intermediate stages of the enzymatic respiratory chain (6) In our study all three behaved alike and are thus regarded as indicators of energy yielding process. The activity



Fig 10 Non specific esterase 10 days after occlusion 100× The lumen of a tertiary bronchus at the top the lining epithelium stains *intensely* Normally all other parts of the lung would be inactive but occlusion has produced active cells close to the bronchus and in the interlobular septum

of the cells in the air blood capillary network around the tertiary bronchi may signify an altered metabolism following the cessation of pulmonary circulation and increase in the bronchial circulation

The occlusion of the pulmonary artery causes a decrease and diffusion of the activity of reductase which may imply disturbances in the active transport mechanisms of the cell membranes (9) Subsequent increase in activity may indicate a proliferative process of the lung tissue The main advantage of this method is the clear demonstration of pulmonary structures

No severe ischaemic or necrotic changes could be observed, either enzyme histochemically or histologically



Fig 11 Naphthylamidase 10 days after occlusion 100× Very intense staining of the epithelium and the walls between the atria of tertiary bronchi



Fig 12 NADH tetrazolium reductase 10 days after occlusion 100× The greatest activity is observed as a *corona* around the bronchial structure The activities of the epithelium and the bronchial muscles are decreased

REFERENCES

1 *Akester A R* The blood vascular system In *D J Bell & B M Freeman* Physiology and biochemistry of the domestic fowl Vol *2* 783–839 Academic Press London 1971
2 *Balogh K Jr Dudley H R & Cohen R B* Oxidative enzyme activity in skeletal cartilage and bone Lab Invest *10* 839–845 1961
3 *Barry D H & Robinson W E* Enzyme histochemical studies on rat lungs 2 Normal enzyme distribution in the alveolar tissue of the mature lung Histochem J *1* 505–515 1969
4 *Burstone M S* Enzyme histochemistry and its application in the study of neoplasms Acad Press New York 1962 pp 395–415
5 *Burton R R Besch E L & Smith A H* Effect of chronic hypoxia on the arterial blood pressure of the chicken Amer J Physiol *214* 1438–1442 1968

6 *Chayen, J, Bitensky, L, Butcher, R G & Poulter, L W* A guide to practical histochemistry Oliver & Boyd, Edinburgh, 1969
7 *Ede, D A* General aspects of the circulation In *D J Bell & B M Freeman* Physiology and biochemistry of the domestic fowl Vol *2* 739–744 Academic Press, London, 1971
8 *French, J E & Benditt, E P* Observations on the localisation of alkaline phosphatase in healing wounds Arch Path *57* 352–356, 1954
9 *Goldfischer, S, Essner, E & Novikoff, A B* The localisation of phosphatase activities at the level of ultrastructure J Histochem Cytochem *12* 72–95, 1964
10 *Gomori, G* Microscopic histochemistry principles and practice Univ Chicago Press, Chicago, 1954
11 *Gould, B S & Gold, N I* Studies on the 'alkaline" phosphatase associated with regenerating connective tissue fibers Arch Path *52* 413–422, 1951
12 *Hosoda, S & Takase, S* Non specific esterase activity in histiocytes Nature (London) *190* 927, 1961
13 *Hunt, R D* Histochemical methods for the demonstration of enzymes In *S W Thompson* Selected histochemical and histopathological methods 615 748 C C Thomas, Springfield, Ill, 1966
14 *Karkola, K & Lansimies, E* Enzyme histochemical studies on the lung of chicken after experimental closure of the left pulmonary artery Scand J clin Lab Invest *29* suppl *122* 48, 1972
15 *Lansimies, E & Valimaki, I* Electrocardiogram in experimental closure of left pulmonary artery in chickens Scand J clin Lab Invest *29* suppl *122* 49, 1972
16 *Nachlas, M M, Crawford, D T & Seligman, A M* The histochemical demonstration of leucine aminopeptidase J Histochem Cytochem *5* 264–278, 1957
17 *Niemi, M & Sylven, B* Intracellular and interstitial enzyme activity in early experimental granulomas Acta path microbiol scand Suppl *187* 76 77, 1967
18 *Pearse, A G E Histochemistry 2nd ed,* Churchill, London, 1960
19 *Raekallio, J* Enzyme histochemistry of wound healing Progress in histochemistry and cytochemistry Vol 1, no 2 Fischer, Stuttgart, 1970
20 *Roh, C, Greene, D, Himmelstein, A, Humphreys, G & Baldwin, E* Cardiopulmonary function studies in a patient with ligation of the left pulmonary artery Amer J Med *7* 795 798, 1949
21 *Weibel, E R* Early stages in the development of collateral circulation to the lung in the rat Res *8* 353–376, 1960

Acta path microbiol. scand Section A 81, 167–173, 1973

RECURRING DIGITAL FIBROUS TUMOUR OF CHILDHOOD

Case Report and Survey

N Grunnet, J Genner, B Mogensen and O Myhre-Jensen

Department of Plastic Surgery, Kommunehospitalet, Århus,
Department of Dermatology, The Finsen Institute, Copenhagen,
Radium Centre and Cancer Research Institute The Danish Cancer Society,
Kommunehospitalet, Århus, and
University Institute of Pathology Kommunehospitalet, Århus

A case of *recurring digital fibrous tumour of childhood* and the results of the patho anatomical and the ultrastructural studies of the tumour are reported The patient was born with a tumour involving the fourth finger of the right hand and, at the age of 10 years she had been treated for 8 recurrences or new tumours on her right, third, fourth, and fifth fingers The case history and the morphological findings indicate a viral origin, but attempts to demonstrate papilloma virus were negative The thirty published cases of recurring digital fibrous tumour of childhood are surveyed So far, excision supplemented with skin grafting has been the standard treatment The best results seem to be obtained by the widest possible excision without damaging the function of the digit Amputation should be considered ultimum refugium Distant metastases have never been observed in spite of the high frequency of local recurrence

Recurring digital fibrous tumour of childhood is a well-defined entity within the heterogenous group of fibromatosis The tumours are present at birth or develop during early childhood and are located to fingers or toes Affected children often have multiple tumours with a high tendency to recur after excision Morphologically the tumours are characterized by intracellular inclusion like bodies (*Reye* 1965) At the moment only 30 cases have been published and so far the lesion has never been observed in adults

We have seen a girl—now ten years old—who was born with this tumour During the course of the disease she has developed eight recurrences or new tumours on the third, fourth, and fifth fingers of the right hand

Received 28 ix 72 Accepted 21 x 72

Requests for reprints should be addressed to N *Grunnet* Blodbanken Kommunehospitalet DK 8000 Århus C Denmark

CASE REPORT

As to the course of the disease the case history falls into 3 distinct periods (a) the fourth finger of the girl was affected at her birth and until she was 6 months old (b) at the age of 4 years the child developed a tumour of the fifth finger, (c) tumours and tumour recurrences involving the third and fourth fingers have appeared since the patient was 8 years old until today (May 1972) when she is 10 years old

(a) A baby girl, otherwise healthy, was born with a soft tumour on the distal phalanx of the right fourth finger (Fig 1, no 1) The tumour slowly enlarged and occupied the ulnar aspect extending distally and dorsally to the nail fold The skin over the tumour was distended and red, but



Fig 1 Recurring digital fibrous tumour of childhood Diagram showing localization and sequence of the tumours in our case (cfr the text)

obviously not tender The movements of the distal fingerjoint was slightly decreased Radiography showed no bone or joint affections The tumour was excised when the patient was 6 months old (b) When the girl was 4 years old, a new tumour was noticed distally on the right fifth digit (Fig 1 no 2) Dislocation of the distal interphalangeal joint and cystic radiolucence in the phalanges were demonstrated by radiography, and the finger soon became deformed A tumour about 'the size of a hazelnut' was removed from the distal phalanx The deformity of the fifth digit progressed, however, and 5 months later the finger was amputated (c) When the child was 8 years old, she developed a tumour on the radial side of the middle phalanx of the fourth finger (Fig 1 no 3), and a tumour mass measuring 5 by 13 mm was excised One month later two recurrences appeared one 10 by 12 mm was located just proximal to the scar (Fig 1 no 4) and the other 8 by 9 mm just distal to it (Fig 1 no 5) Furthermore a new tumour developed on the ulnar aspect of the middle phalanx of the right fourth digit (Fig 1 no 6) The 3 tumours were all fairly dense, not tender, and covered with red skin A few months later, a tumour developed on the ulnar aspect of the middle phalanx of the third finger (Fig 1 no 7) just opposite to one of the tumours on the fourth finger At the same time, the skin became indurated on the volar aspect of the proximal phalanx of the third right finger in an area of 8 by 10 mm (Fig 1, no 8) These 5 tumours were removed within a few months. About the time of operation, radiography of the right fourth finger showed osteolytic defects in the capitulae of the proximal and the middle phalanges and sclerosis in the distal phalanx Serum alkaline phosphatases were moderately increased (42 0 and 44 2 units normally 5-15) These changes had regressed a few months later Finally, some months after the last operation, a tumoursuspect process (Fig 1, no 9) appeared on the ulnar and volar side of the proximal phalanx of the third finger close to the interphalangeal joint This process is still present and has remained unchanged during the last 9 months When the girl was seen last (May 1972) and 15 months earlier, samples of serum were examined for complement fixing antibodies against papilloma virus (*Genner* 1971 b) with negative result

PATHO-ANATOMY

Twelve tissue specimens from tumour excisions including the amputation, were examined The tissue was removed over a period of 9 years Most specimens were skin covered and contained a varying amount of subcutaneous tissues On the cut surface the tumours were whitish, whorled and rather dense The tumours, removed when the girl was a few months old, were more cellular and contained more mitotic figures than later tumours Otherwise, the histology of all the tumours was identical The tumour processes were confined to the corium and usually extended to a certain degree into the subcutaneous tissue The epidermis was slightly hyperplastic and hyperkeratotic but otherwise normal The tumours were not circumscribed or encapsulated (Fig 2) At the periphery skin appendices were surrounded and partly compressed by the tumours the central parts of which contained no sweat glands The tumours were composed of fibroblasts and collagen fibrils in a coarsely woven network of cells and fibrils differing but little from the normal dermal connective tissue The amount of elastic tissue however had decreased At the periphery small infiltrates of lymphocytes and plasmacells could be seen and normal nerves and tactile bodies were encountered but nerve fibres could not be detected within the tumours There was no palisading of cells and no cart-wheel appear-



Fig 2 Recurring digital fibrous tumour of childhood cross section of tumour (Fig 1 no 5) h–e × 15 The tumour is confined to the corium extending slightly into the subcutaneous tissue The epidermis is intact

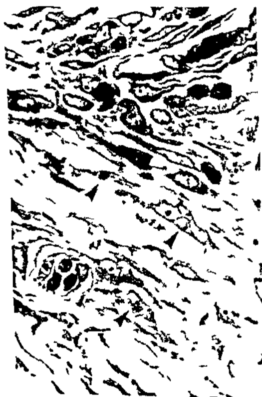


Fig 3 Recurring digital fibrous tumour of childhood semi thin section of Epon embedded osmium fixed tissue (Fig 1 no 5) Toluidine × 1250 Three intracytoplasmic inclusion like bodies one indenting a cell nucleus are indicated by arrows

ance Xanthoma cells and Touton giant cells were absent

Round inclusion like bodies 5 10 nm were found in all the tumours Usually they were located in the cytoplasma of the fibroblast and sometimes they indented the nucleus (Fig 3) The bodies appeared hyalinous in the haematoxylin and eosin stained sections and darkblue in phosphotungstic acid – haematoxylin stained sections

ULTRASTRUCTURE STUDIES

Parts of one of the last tumour recurrences (Fig 1 no 5) was fixed in osmium tetroxide dehydrated in acetone Epon embedded and studied with the electron microscope

The cells of the tumour were elongated with cytoplasmic processes containing a fairly rich amount of endoplasmic reticulum some

TABLE 1 *Survey of 30 Published C*

Author	Sex	Age at diagnosis	Localization at diagn
Sakurane 1924 (Japan)	n i	2 yrs	Right 3rd and 4th f Left 2nd and 3rd fin
Kapiloff and Prior 1952 (USA)	F	2 yrs	Right 3rd finger
Prior and Sisson 1954 (USA)	M	2 3 mos	Right 3rd and 4th f
Stout 1954 (USA)	M M M	3 mos at birth 1 mo	Left 4th and 5th, fin Right 3rd toe Left 2nd toe Right 4th toe
Jensen et al 1957 (USA)	4 F 2 M	average 2½ mos	Toes and fingers (In all cases but one t distal phalanx was invo
Booher and McPeak 1959 (USA)	F	1 yr	Left 4th finger
Enzinger 1965 (USA) *	F	4 mos	Right 4th finger
Reye 1965 (Australia)	M F M M F F	at birth n i 4 mos at birth n i 9 mos	Toe Fingers 2 tumours) Finger 3rd toe Finger Fingers (3 tumours)
Ahlquist et al 1967 (Finland)	F	at birth	Left 3rd finger
Shapiro 1969 (USA)	M	at birth	Right 2nd toe
Burry, Kerr, Pope 1970 (Australia)	F	3 mos	Right 4th finger
Battifora and Hines 1971 (USA)	F	3 mos	Left 5th finger
Grunnet, Genner, Mogensen & Myhre Jensen 1972 (Denmark)	F	at birth	Right 4th finger

n i no information given
* Enzinger reported on 6 cases, which were obviously similar A detailed case history was given in one of these cases only

mitochondria and microbodies Nuclei were regular, each containing one single nucleolus The cells were surrounded by heavy bundles of collagen fibrils in a fairly regular pattern parallelling the elongated cells Several large inclusions were seen in the cytoplasma of the fibroblasts usually as dense, well demarcated bodies surrounded by a membrane, others with poorly defined outlines The interior was finely granular (Fig 4) Some inclusions contained small, well demarcated, dense bodies In close proximity to some of the inclusions small tubules or vesicles were found No inclusions were seen in the epidermis

Furthermore, another part of the same tumour specimen was prepared for papilloma virus identification according to the technique previously described (*Genner* 1971 a) Electron micrographs were obtained from uranyl acetate (2 per cent) and ammonia molybdate (2 per cent) stained material Numerous collagen fibrils and several ovoid

of recurrences	Treatment and follow up
odules were found the toe	Excision The nodules increased in number, leaving free only the thumbs
	Excision at 2½ yrs Re-excision at 4 yrs No further information
	Excision at 8 mos Re-excision at 12 mos Recurrence at 15 mos Amputation of both fingers in metacarpalphalangeal joints
ı	5th finger amputated Excision on the 4th Not followed
	Excision at 5½ mos. Re excision, age 6 mos. A second recurrence was removed 11 mos later
	Amputation, 2nd left toe, age 6 mos. 4th right toe, age 2 yrs Followed for 10 yrs Tumour extends to the foot, but the patient walks well
l in 3 cases,) in 1 case	Excision, later amputation
l other tumours	Excision at 27 mos The patient died postoperatively of acute tracheobronchitis
2	Excision High recurrence rate seems to be the rule
n ı	Amputation at 7 mos
n ı	*Excision, age 4 yrs*
n ı	Excision age 10 mos
I	Amputation, age 10 mos. No recurrences after 2 yrs
n ı	Excision, age 3½ yrs
n ı	Excision, age 1 yr
3	Amputation of 2 distal phalanges of 3rd and 5th fingers, age 6 mos At 8–9 mos tissue was removed from the scars
0	Excision No other lesions after 5 yrs
1	Excision at 5 mos Re excision widely, few weeks later
5	Excision Re excision No recurrences 4 mos later
8	Excision Amputation of 5th right finger At the age of 10 yrs she has a tumour suspect process on 3rd right finger

or spherical bodies, 100–200 nm, without structure of the interior were found (Fig 5) The ovoid or spherical bodies probably represent cell constituents Papilloma virion *inclusions* were not identified

DISCUSSION

The survey of the 30 *published cases of re*curring digital fibrous tumour of childhood (Table 1) clearly demonstrates the clinical characteristics of the disease (a) presence at birth or development in the very early childhood, (b) localization to fingers or toes, (c) often multiple occurrence and (d) a high tendency to recurrence

Since *Reye's* discovery (1965) of the intr cytoplasmatic inclusion-like bodies this hig specific histological feature has further ser *to distinguish the tumour from other f*i matoses in childhood Actually, *Reye* con the name "*recurring digital fibrous*



Fig 4 Thin section of osmium fixed Epon embedded and uranylacetate stained material (Fig 1 no 5) × 29000 An intracytoplasmic inclusion like body granular, with poorly defined outlines is demonstrated



Fig 5 Saline homogenate of tumour (Fig 1, no 5) Negative stain with 2 per cent uranylacetate, × 80000 (instrument magnification × 40000) The spherical bodies demonstrated probably represent cell constituents (The photograph was taken in the physical laboratory II of the H C Ørsted Institute, University of Copenhagen)

childhood Previously the following terms had been used dermatofibroma, neurofibrosarcoma, dermal fibromatosis, infantile digital fibromatosis, or infantile dermal fibromatosis

The tendency to recurrence or spread to adjacent fingers which is so well demonstrated in our case, caused *Jensen et al* (1957) to suggest a viral aetiology, which suggestion was further supported by the demonstration of cellular inclusions (*Reye* 1965) The attempts of *Pohjanpelto et al* (1967) to culture the viral agent failed and later attempts to isolate a virus were equally unsuccessfull (*Burry, Kerr and Pope* 1970) However, the studies by electron microscope in the case published by *Battifora and Hines* (1971) supported the theory of a viral origin

These investigators found amorphous and fibrillary inclusions of an appearance similar to that of viroplasm of fibroblasts infected with Shope fibroma virus In our study a similar finding was made

Bovine papilloma virus is accused of causing a fibropapillomatous, benign tumour of equine skin Neither human papilloma (wart) virus nor complement fixing antibodies however were demonstrated in our case Thus, a vir [illegible]

sai [illegible]

spherical bodies found in our study (Fig 5) are unknown They probably represent cell constituents

Although the tumour often recur, its growth rate is usually slow So far, excision supplemented with skin grafting has been

standard treatment The best results seem to be obtained by the widest possible excision without damaging the function of the digit Amputations should be considered ultimum refugium The rather conservative attitude is justified by the observations by *Stout* (1959) who followed a boy with an untreated tumour for 10 years Although the tumour persisted, no distant metastasis occurred

REFERENCES

Ahlquist J, Pohjanpelto, P, Hjelt, L & Hurme, K Recurring digital fibrous tumour of child hood 1 Clinical and morphological aspects of a case Acta path. microbiol scand *70* 291–296, 1967

Battifora, H & Hines J R Recurrent digital fibromas of childhood An electron microscope study Cancer *27* 1530–1536, 1971

Booher, R J & McPeak, C J Juvenile aponeurotic fibromas Surgery *46* 924–931, 1959

Burry, A F Kerr, J F R & Pope, J H Recurring digital fibrous tumour of childhood An electron microscopic and virological study Pathology *2* 287–291, 1970

Enzinger, F M Fibrous tumour of infancy In Tumours of Bone and Soft Tissue Year Book Medical Publ, Inc, Chicago 1965, p 375–379

Genner, J Localization of wart virus antigen in human plantar warts (verrucae vulgares) Acta Dermato-Venerol. *51* 351–364, 1971 a

Genner, J Verrucae vulgares II Demonstration of a complement fixation reaction Acta Dermato-Venerol *51* 365–373, 1971 b

Jensen, A R, Martin, L W, Longino, L A Digital neurofibrosarcoma in infancy J Paediatrics *51* 566–570, 1957

Kapiloff, B & Prior, J T Fibromatosis in children Plastic and Reconstructive Surgery *10* 276–282, 1952

Pohjanpelto, P Ahlquist, J, Hurme, K & Hjelt, L. Recurring digital fibrous tumour of child hood 2 Isolation of a Cell transforming Agent Acta path microbiol scand *70* 297–299, 1967

Prior, J T & Sisson, B J Dermal and fascial fibromatosis Annals of Surgery *139* 453–467, 1954

Ragland, W L., Keown, G H & Spencer, G R Equine sarcoid Equine Vet J *2* 2–11, 1970

Reye, R D K Consideration of certain sub dermal 'fibromatous tumours of infancy J Path Bact *72* 149–154, 1966

Reye, R D K Recurring digital fibrous tumours of childhood Arch. Path *80* 228–231, 1965

Sakurane, K A case of fibroma durum multiplex on the tip of the fingers and toes of an infant Japanese Journal of Dermatology *24* 8, 1924

Shapiro H L Infantile digital fibromatosis and aponeurotic fibroma Acta Derm *99* 37–42, 1969

Stout, A P Juvenile fibromatosis Cancer *7* 953–978, 1954

Acta path microbiol scand Section A 81, 174–182 1973

HYPERTROPHIC OBSTRUCTIVE CARDIOMYOPATHY

A Review of the Patho-Anatomic Findings and the Clinical Characteristics Including a Report of Two Additional Cases

JØRN SIMONSEN, JØRGEN VOIGT and KJELD LYNGBORG

The University Institutes of Forensic Medicine Copenhagen and Odense, and Medical Department B Rigshospitalet, Copenhagen

Two cases of hypertrophic obstructive cardiomyopathy (HOCM) seen among a total of *11 141 medico-legal autopsies are reported* A review of the literature is *presented It shows* that the disease occurs rarely and probably is hereditary The manner of transmission is not known Pathoanatomically the diagnosis of HOCM ought to be based on macroscopical as well as microscopical findings In the view of the authors the greater weight should be placed on the macroscopical findings as the microscopical abnormalities are not necessarily pathognomonic and vary in incidence The marked and dominant hypertrophy of the ventricular septum is the characteristic finding in this disease The cause of death is often congestive heart failure but frequently death is sudden and unexpected

Sudden death from cardiac disease is most often accounted for by defective coronary circulation resulting in myocardial ischaemia Ranking second among the causes are congenital or acquired valvular disorders and third, asserting themselves more rarely, various congenital heart diseases and myocardiopathies including myocarditis To the latter group belongs hypertrophic obstructive cardiomyopathy (HOCM), which pathoanatomically is characterized by marked asymmetrical hypertrophy mainly affecting the interventricular septum Considering that recognition of HOCM as a disease entity is of fairly recent date and that the disease may run a symptomless course until death suddenly occurs, this article intends to give a review of the disease as well as report two cases of sudden death due to HOCM

Received 19 ix 72 Accepted 24 x 72

Requests for reprints should be addressed to *Jørn Simonsen*, University Institute of Forensic Medicine, J B Winsløwsvej 15, DK 5000, Odense, Denmark

LITERATURE

It is generally recognized that HOCM was first mentioned in the literature by *Schminck* (1907) who reported two cases of hypertrophy of the left ventricle mainly involving the outflow area The description went unnoticed

In the late 1950's two papers on HOCM appeared almost simultaneously *Brock* (1957) on the basis of surgical and autoptic findings described a case of functional aortic stenosis due to muscular hypertrophy In his opinion now shared by few the hypertrophy was caused by hypertension *Teare* (1958) gave a detailed pathoanatomical description of the disease based upon eight cases collected among 16 000 autopsies In his description to which very little has since been added, he drew attention to the macroscopical finding of asymmetrical septal hypertrophy Microscopically he found muscle fibres extending irregularly in various directions often broken up by considerable amounts of collagenous connective *tissue and clefts He interpreted* these abnorma

lities as being in the nature of massive muscular heart tumour Moreover, he pointed to the typical clinical picture (sudden death of young persons) and demonstrated the existence of a hereditary factor, three of the subjects in his study were siblings

Brock and *Teare* published their descriptions at a time when catheterization of the left side of the heart had become feasible This fact, in conjunction with the introduction of extracorporeal circulation rendering left sided heart surgery possible, attracted increased attention to HOCM, which has since been the subject of several articles and two international symposia

In the pathoanatomical field, studies of large series by, among others, *Pearse* (1964), *Marshall* (1970), *Snijder et al* (1970), *van Noorden et al* (1971), and *Olsen* (1971) have had little to add to the original description given by *Teare* (1958) The chief characteristic of HOCM is massive hypertrophy of the ventricular septum, particularly of its upper part beneath the anterior aortic valve This change is best revealed by a special tecnique of heart dissection involving cross section of the heart at right angles to the axis (Incidentally, this technique is by far the one to be preferred in general) Other characteristics are disorderly arrangement of muscle fibres macroscopically as well as microscopically, with massive hypertrophy of the individual fibres (diameters of up to 90 μ have been reported), and large, irregularly shaped nuclei with perinuclear haloes In typical cases the muscle fibres are broken up irregularly by streaks of collagenous connective tissue Recent finding is whorl formation by muscle bundles (*van Noorden et al* 1971, *Olsen* 1971)

Van Noorden et al (1971) established a so-called histological HOCM index by registering the presence in preparations of

(1) short fibres broken up by connective tissue,
(2) large, bizarre nuclei,
(3) fibrosis,
(4) muscular degeneration in the form of myofibrillar atrophy,
(5) disorganization and whorl formation

Scores ranging from 0 to 3 were given for each of these items, with 15 as the maximum obtainable score

For many years, electron microscopy contributed only to confirm findings previously made by light microscopy But in a recent study by *Ferrans et al* (1972), electron microscopy disclosed abnormalities additional to those seen in other types of myocardial hypertrophy The authors saw fibrils extending in various directions, shorter and wider muscle cells, frequent side-to-side junction of muscle cells, z bands of increased width, and formation of new sarcomeres.

Histochemically, the glycogen content is always higher than in control preparations, whereas enzymatic activity is normal (*Snijder et al* (1970)) In ischaemic myocardial tissue, by contrast, enzymatic activity and glycogen content are both very low Unlike in alcoholic cardiomyopathy, fat deposits in cells are practically never seen

Van Noorden et al (1971) failed to produce conclusive evidence of the findings recorded by *Pearse* (1964) of increased noradrenaline activity in the hearts of patients with HOCM and increased amounts of peripheral nerve tissue Further research will be required before any definite conclusion can be reached

It is thus maintained that the diagnosis of HOCM, to the exclusion of other cardiac disorders, can be made with a reasonable degree *of certainty on the basis of the histological* findings alone As the changes occur focally and are not pathognomonic, a fairly extensive biopsy will be required This alone will limit *the use of myocardial biopsy in the diagnosis* of this disease

The aethiology of HOCM has not yet been definitely established As familial incidence has been reported in approximately 25 per cent of subjects studied (*Emanuel* 1971), and as cases have been seen even in infants (*Daoud et al* 1961, *Shand et al* 1971), the disease is probably hereditary The manner of transmission remains to be established

The symptoms, usually brought on by physical exertion, are dyspnoea, angina pectoris, palpitations, vertigo and perhaps syncope However, the disease may run a symptomless course On the whole, the *symptomatology does not differ from that of valvular* aortic stenosis Objective findings are not identical, however, the murmur heard in HOCM is loudest at the lower left sternal border, while the murmur rarely radiate to the neck In HOCM *the pulse is typically jerky and collapsing (celer)*, whereas in valvular aortic stenosis it is usually slow rising and sustained (tardus) In both diseases there may be a faint murmur of aortic insufficiency present, and there may be signs of left sided and right sided heart failure ECG will usually indicate the presence of severe, left ventricular hypertrophy and septal Q waves (*Frank and Braunwald* 1968) Roentgenograms of the chest will often reveal enlargement of the left ventricle The ascending aorta unlike in valvular aortic stenosis, will not be dilated Pulmonary stasis may be present

Clinically, the diagnosis can be made by left sided catheterization and angiocardiography Catheterization will show a systolic pressure gradient between the left ventricle and the aorta. This

gradient is located subvalvularly, and is increased after extra systoles (*Brockenbrough et al* 1961) Approximately 25 per cent of cases will not show any gradient at rest (*Harrison et al* 1964), but it can usually be produced by isoprenalin (a beta adrenergic stimulant) Drugs blocking the beta effectors of the sympathetic nervous system will similarly reduce pressure differences in less severe cases (*Kochsiek et al* 1971) Another finding, depending on the degree of severity of the disease, is increased diastolic pressure in the left ventricle Moreover, there may be a large a wave in the left atrium, indicative of the increased stiffness of the left ventricle (*Kochsiek et al* 1971) Angio cardiography will show obstruction of the outflow area during the mid and endsystole, caused by approximation of the interventricular septum and the anterior mitral leaflet Hypertrophy of the septum and the adjacent portion of the anterior wall of the left ventricle is typical (*Bevegård et al* 1962, *Nordenstrom and Ovenfors* 1962, *Simon et al* 1967) Mitral insufficiency during late systole has been recorded in 93 per cent of subjects in a recent study (*Flamm et al* 1967)

The prognosis is not yet definitely established as studies of large series with long follow ups are not available Within a period of three years 10 out of 98 patients died from cardiac disease, six of them suddenly, oddly enough, only one of them had a significant pressure gradient (*Frank and Braunwald* 1968) In this connection it is worth mentioning that 10 out of 16 subjects studied by *Marshall* (1970) did not have any symptoms prior to their sudden deaths Bacterial endocarditis is a known complicating factor affecting aortic as well as mitral valves (*Frank and Braunwald* 1968)

Medical treatment may be attempted by drugs blocking the beta effectors of the sympathetic nervous system so as to reduce pressure differences between left ventricle and aorta lower the frequency of arrhythmia and, consequently, reduce the risk of sudden death The effect of such treatment on prognosis is at yet not known Surgical treatment by myotomy and/or resection will usually eliminate or reduce a pressure gradient (*Morrow et al* 1968) Following this treatment the patients are not cured, however, since the muscular hypertrophy is not completely removed

CASE REPORTS

Case No 1 (RH, B 1231/64–65, R I, D 166/65)

This patient was first hospitalized at the age of 16 because of fainting spells and abnormal ECG There was no family history of cardiac disease He had always, he thought, been short of breath for which reason, among others, he had never played football His condition was no worse, however, than he could walk up to the tenth floor During



Fig 1 Case No 1 Septum with interstitial fibrosis Right ventricle upward, left ventricle downward

the six months preceding his hospitalization he had had three fainting spells, two of them following exertion For this reason he was admitted to the department of neurology As the neurological findings were negative he was transferred to our cardiological department for further examination

On physical examination the patient was found to be slightly short for his age Neither cyanosis nor dyspnoea was present and there was no signs of rightsided or leftsided heart failure On stethoscopy a systolic murmur was heard over the entire precordial area, loudest over the third left intercostal space, where it was of intensity 3 and of ejection type There was no radiation of the murmur to the neck No diastolic murmur was



Fig 2 Case No 1 Ventricular septum viewed from right ventricle Note the trabecular hypertrophy



Fig. 3. Case No. 1. Ventricular septum with disordered musculature. Viewed from left ventricle.

heart. ECG was pathological showing Q waves in standard leads II and III and in precordial leads V_{4-6} which were 1.1–1.5 mV deep and 0.03 sec. wide. Roentgenograms of the chest showed a normal-sized heart (370 cc/m²) but prominence of the left ventricle. The pulmonary vasculature was normal. Right-sided cardiac catheterization revealed normal pressures and ruled out intracardiac shunt. The conclusion of the examinations was that the patient was suffering from a cardiac disease, the type of which could not, however, be precisely defined, and the patient was discharged from hospital.

The patient was followed in the outpatient department and during two subsequent hospitalizations, two and six years later respectively. Two years after his first discharge he felt slight precordial pain on exertion, but did not faint. ECG and roentgenogram of the chest showed no definite changes over the follow-up period. During his last hospital-admission, left-sided catheterization was performed retrograde from the *femoral artery*; no aortic gradient was demonstrated. The diastolic pressure in the left ventricle was within the upper normal range (14 mm Hg). Angiocardiography was not performed.

Shortly after the last examination he was found dead early in the morning, lying on the road beside his bicycle. There was nothing to indicate that he had been involved in a traffic accident. At the time of his death the patient was 22 years old.

At medico-legal autopsy the only external signs of violence noticed were a few abrasions on cheeks and hands. The body weight was 70 kg. The heart weighed 5[illegible]0 g and measured 13 × 11 cm externally; it was almost globular. Upon incision, the most conspicuous finding was massive hypertrophy of the left ventricular septum, the maximum thickness being close to 5 cm (Fig. 1). The thickness of the wall of the left ventricle was only 12 mm. On the right side, the ventricular wall measured 5 mm at the thickest point (Fig. 2). There was marked hypertrophy of the trabeculae of both ventricles (Figs. 2 and 3). No narrowing of great vessels or valves was seen, neither were septal defects. The coronary arteries showed no abnormality. Macroscopically, no abnormality was noticed on incision of the myocardium.

Apart from the heart disease the autopsy did not reveal any abnormality.

Macrodissection of muscle bundles showed the deep fibres disorderly intermeshed.

The microscopical findings are recorded in tabular form, see pagina [illegible].

Case No. 2 (RH, B 1244/60–67, RJ, D 38/71)

This patient was at the age of 14 hospitalized for a systolic murmur noticed in the course of a routine examination at school.

Family history. A paternal uncle suffering from muscular dystrophy was reported to have a coronary thrombosis, the maternal grandmother had had a coronary thrombosis at the age of 60, a brother died at the age of six months following an infectious disease.

The patient had no previous history of dyspnoea, precordial pain or fainting. He was found on physical examination to be normally developed, neither cyanosis nor dyspnoea was present. There was no clinical evidence of heart failure. Stethoscopy revealed ictus cordis to be located in the fifth intercostal space, lateral to the medioclavicular line. A systolic murmur was heard over the entire precordial area, loudest at the apex where it was of intensity 4/6 and of ejection type. The murmur radiated to the neck. Over the apex a short, rough protodiastolic murmur of intensity 1/6 was also heard. Radial and femoral pulses were of celer type. BP 120/60. ECG showed a left-sided hypertrophic pattern with a Q wave in lead III. X-ray of the chest revealed slight enlargement of the heart (425 ml/m²); especially the left ventricle appeared to be enlarged. The pulmonary vasculature was normal. Right-sided catheterization ruled out intracardiac shunt. Subvalvular pulmonary stenosis was seen, the gradient being 32 mm Hg. Left-sided cardiac catheterization by means of atrial septal puncture and retrograde catheterization of the left ventricle from the femoral artery revealed, in non-simultaneous curves at rest, the presence of a subvalvular aortic gradient of 42 mm Hg. After extra-systoles the pressure in the left ventricle rose from 127 to 23[illegible] mm Hg. Angio-cardiography using injection of contrast medium into the left atrium showed massive hypertrophy of the interventricular septum and the presence of stenosis in the outflow area of the left ventricle. It could thus

Table of Histological Findings

	1	2
General description		
Structure	Somewhat coarse and irregular	Somewhat coarse and irregular
Fibrosis	Considerable, *irregular*	Considerable, *irregular*
Acute changes		
Inflammation	Sporadic, slight infiltration of lymphocytes	0
Necrosis	0	0
Vascular changes	0	Slight arteriolar sclerosis
Pericardium	No abnormality	No abnormality
Endocardium	Slightly thickened	Slightly thickened
Elastic tissue	No abnormality	No abnormality
Glycogen	Some	0
Cross-striation	Intact	Intact
Nerves	No abnormality	No abnormality
Diameters of muscle fibres	10–30 μ	10–30 μ
Index (calculated *ad modum van Noorden et al*, 1971)		
1) Short fibres broken by connective tissue	2	2
2) Large, bizarre nuclei	3	2
3) Fibrosis	2	3
4) Muscular degeneration with atrophy of myofibrils	0	0
5) Disorganization, whorls	1	1
Index totals	8	8
Index in per cent of maximum score	53 per cent	53 per cent
Sinus node and atrioventricular system	Questionable fibrosis of sinus node otherwise no abnormality	Slight fibrosis of atrioventricular bundle otherwise no abnormality

Microscopical examination was made of samples of myocardium removed from the anterior and posterior walls of the left ventricle and from the interventricular septum. In case No. 2 tissue was also removed from a large papillary muscle. Systematic examination of sinus nodes and atrioventricular systems were performed by means of serial sections. Stains employed were: connective tissue stain (sirius red/celestine blue), elastin stain (orcein), PAS and Best's carmine stain for glycogen content, *PTAH* for cross-striation, ethylamine-silver oxalate for nerve fibers in tissues.

be established that the patient suffered from hypertrophic cardiomyopathy with subvalvular aortic stenosis and pulmonary stenosis. Propanolol treatment, 10 mg q.i.d. was tentatively instituted. Following control catheterization which revealed no reduction of the pulmonary gradient, treatment was discontinued and the patient discharged. He was advised to avoid competitive sports and to find a job requiring little physical exertion.

The patient was followed in the outpatient department. Two years after his discharge he fainted after having tried to catch a bus by running fast.

Fig 4 Case No 2 Cross section of ventricles Note the asymmetrical hypertrophy

On subsequent examination slight increase of the left ventricular hypertrophy was recorded as well as a more marked prominence of the left ventricle, was seen on the roentgenogram of the chest. Propanolol treatment, 10 mg q i d, was reinstituted, despite of it the patient fainted twice following exertion (running) At the age of 18 he suddenly collapsed after climbing stairs to a platform at a building site where he was engaged in work requiring little physical effort On arrival to the hospital he was dead

toma
kg
cm
ntri
(Fig 4)

The wall of the right ventricle was up to 8 mm thick that of the left ventricle up to 20 mm There was marked prominence of the left ventricular septum occupying the upper region of the left ventricle beneath the aortic valves Otherwise, there was no abnormality of valves, coronary arteries and great vessels A small firm area was found in the lower lobe of the right lung, probably a minor infarct

The microscopical findings are recorded in the table on p 5

12*

DISCUSSION

The above described pathoanatomical findings are characteristic, but not necessarily pathognomonic (*Teare* 1958, *Pearse* 1964, *Marshall* 1970, *Snijder et al* 1970, *van Noorden et al* 1971, *Olsen* 1971, *Oakly* 1971) The latter expressed the opinion that while histochemical and ultrastructural methods of examination have proved to be disappointing, conventional histology permits differential diagnosis between HOCM, congestive cardiomyopathy and "normal" secondary hypertrophy due to aortic stenosis In the study reported in this paper the most marked changes were found in the highly hypertrophic septum, see the table on p 178 The aforementioned system of scoring devised by *van Noorden et al* (1971) may some times give an idea as to whether the abnormalities observed are attributable to HOCM or to some other type of cardiomyopathy In



Fig 5 Case No 1 Myocardium from interventricular septum. Note the whorled structure (sirius red/celestine blue stain, × 56)



Fig 6 Case No 2 Myocardium from interventricular septum Note the marked interstitial fibrosis (connective tissue black coloured) (sirius red/celestine blue stain, × 56)

our two cases, however, the changes were not particularly marked, and they did in no way seem to be specific It is our impression that *similar* changes may be seen in other cases of severe cardiac hypertrophy with resulting hypovascularization Using the scoring system we obtained an index which did not provide any reliable guide, it was clearly within what *van Noorden et al* (1971) referred to as the area of overlapping We found, therefore, that in our two cases the diagnosis of HOCM could not be established on the basis of the histological findings alone

We could not with certainty, determine the glycogen content since the histological preparations were made from tissue fixated in formaldehyde It was difficult to demonstrate presence of the whorls described by *van Noorden et al* (1971), and we could not clearly see the haloes around the nuclei reported by these authors As the more specific histochemical methods reported by others did not appear to contribute in the evaluation, we have accordingly decided not to evaluate them further

Accordingly in our opinion the pathoanatomical diagnosis of HOCM ought to be based primarily on the macroscopical findings

Teare (1958), found 8 cases of HOCM among 16,000 autopsies, *Marshall* (1970) reported an incidence of one in every 200 cases of sudden cardiac death The two cases reported in this paper were seen over a period of twelve years in which Institute of Forensic Medicine performed a total of 11,141 autopsies Thus, the incidences reported vary considerably No direct comparison can be made, however, since the figure given by *Marshall* (1970) is related to the total number of cardiac deaths while the figures given by *Teare* (1958) and in our material are related to the total number of autopsies It may often be difficult to differentiate HOCM from a pronounced concentric hypertrophy also involving the outflow area of the left ventricle Another *factor affecting the statis*tics are the criteria for performing autopsy in cases of death from natural causes Furthermore, a considerable number of cases may not have been reported to the institutes of forensic medicine, as hospitalization due to non specific symptoms might have occurred prior to death Finally it is essential that the forensic or hospital pathologist is familiar with the existence of this disease entity It is, therefore not surprising, that the number of reported cases of HOCM has increased considerably after *Brock* (1957) and *Teare* (1958) published their studies An incidence of 8 cases of HOCM among 56 patients with aortic stenosis was reported in a clinical study by Hansen (1967)

The cause of sudden death of patients with HOCM has not been established with certainty One hypothesis is sudden increase of the subvalvular stenosis susceptible as it is to the influence of the sympathetic nervous system or sudden failure of the left ventricle

with resulting hypotension and myocardial ischaemia This hypothesis is hard to prove or disprove However, one case has been reported of near syncope brought on by physical exertion and accompanied by marked hypotension (*Flamm et al* 1967) An alternative explanation is sudden occurrence of arrhytmia, notably ventricular fibrillation and ventricular tachycardia, but asystole is another possibility Such rhytmic disturbances have been observed in patients with valvular aortic stenosis, one case of death from ventricular fibrillation in HOCM has been reported (*Schwartz et al* 1969) As marked hypertrophy of the left ventricle and fairly high incidences of syncope and sudden death are features common to valvular aortic stenosis and HOCM, the mechanism of death is probably the same in both diseases Actually, one case of ventricular fibrillation in HOCM has been reported (11) Interplay of the above mentioned factors is another likely explanation It remains uncertain whether physical exertion can provoke death of patients with HOCM Both patients in our study apparently died following exertion (cycling, climbing stairs) *Marshall* (1970) found that only 3 out of 13 patients had been subject to any exertion (walking) prior to their sudden deaths. On this basis he concluded that exertion is not a precipitating factor

Addendum

After finishing of the manuscript we have observed one more case of HOCM at the University Institute of Forensic Medicine of Copenhagen

This was a 22 year-old narcotic addict who died from an overdose of morphine There were no anamnestic information at all

The heart was typical, 580 g/84 kg

The frequency of HOCM in the Copenhagen Institute is after this 3 cases in 11 568 autopsies i e 1 case per just under 4 000 autopsies.

REFERENCES

1 *Bergård S Jonsson, B & Larlof, L* Low Subvalvular Aortic and Pulmonic Stenosis Caused by Asymmetrical Hypertrophy and Derangement of Muscle Bundles of the Ventricular Wall. Acta med Scand *172* 269–*283 1962*

2 *Brock, R* Functional Obstruction of the Left Ventricle Guy's Hosp Rep *108* 221–283, 1957

3 *Brockenbrough, E C, Braunwald, E & Morrow, A G* Hemodynamic Technic for the Detection of Hypertrophic Subaortic Stenosis Circulation *23* 189–194, 1961

4 *Daoud, G, Gallaher, M E & Kaplan, S* Muscular Subaortic Stenosis Am J Cardiol *7* 860–864, 1961

5 *Emanuel, R* Hypertrophic Obstructive Cardiomyopathy In Ciba Foundation *Study* Group no 37 *G E Wolstenholme & M O Connor*, Editors, J & A. Churchill, London 1971 (p 50–54)

6 *Ferrans, V J, Morrow, A G & Roberts, W C* Myocardial Ultrastructure in Idiopathic Hypertrophic Subaortic Stenosis Circulation *45 769–792, 1972*

7 *Flamm, D, Braniff, B A, Kimball, R & Hancock, E W* Mechanism of Effort Syncope in Aortic Stenosis. Circulation *35 suppl 12* 109–109, 1967

8 *Frank, S & Braunwald, E* Idiopathic hypertrophic Subaortic Stenosis. Circulation *37* 759–788, 1969

9 *Hansen, P F* Aortic Stenosis. Thesis, Munksgaard, Copenhagen 1967

10 *Harrison, D C, Braunwald, E, Glick, G, Mason, D T, Chidsey, L A & Ross, J* Effects of Beta Adrenergic Blockade of the Circulation with Particular Reference to Patients with Hypertrophic Subaortic Stenosis Circulation *29* 84–98 1964

11 *Hilty Tammivaara, R & Culhed J* Excercise Related to Ventricular Fibrillation in Hypertrophic Subaortic Stenosis Treated by Propanolol Acta med Scand *187* 317–322 1970

12 *Kochsiek, K Larbig D & Harmjanz, D* Die hypertrophische obstruktive Kardiomyopathie Springer Verlag Berlin (p 68), *1971*

13 *Marshall T K* Asymetrical Hypertrophy of the Heart Med Sci Law *10* 3–6 1970

14 *Morrow A G Fogarthy T J Hunger, H & Braunwald E* Operative Treatment in Idiopathic Hypertrophic Subaortic Stenosis Circulation *37* 589–596 1968

15 *van Noorden S Olsen E G J & Pearse, A G E* Hypertrophic Obstructive Cardiomyopathy Cardiovasc Research *5* 118–131, 1971

16 *Nordenstrom B & Ovenfors, C O* Low Subvalvular Aortic and Pulmonic Stenosis with Hypertrophy and Abnormal Arrangement of the Muscle Bundles of the Myocardium Acta Radiol (Scand) *57* 321 340 1962

17 *Oakley C* Ventricular Hypertrophy in Cardiomyopathy Brit Heart J *33 suppl* 179–186, 1971

18 *Olsen, E G J* Morbid Anatomy and Histology in Hypertrophic Obstructive Cardiomyopathy Ciba Foundation Hypertrophic Obstructive Cardiomyopathy J & A Churchill, London 1971 (p 183 191)

19 *Pearse, A G E* The Histochemistry and Electron Microscopy of Obstructive Cardiomyopathy Ciba Foundation Cardiomyopathies J & A Churchill, London 1964 (p 132, 171)

20 *Schmincke, A* Ueber linksseitige muskulose Conusstenosen Dtsch med Wschr *33* 2082–2083 1907

21 *Schwartz L S, Goldfischer, J, Sprague, G J & Schwartz, S P* Syncope and Sudden Death in Aortic Stenosis Am J Cardiol *23* 647–658, 1969

22 *Shand, D G, Sell, C G & Oates, J A* Hypertrophic Obstructive Cardiomyopathy in an Infant Propanolol Therapy for Three Years New Engl J Med *285* 843 844, 1971

23 *Simon, A L, Ross, J & Sault, J H* Angiographic Anatomy of the Left Ventricle and Mitral Valve in Idiopathic Subaortic Stenosis Circulation *36* 852–867, 1967

24 *Snijder, J, de Jong, J & Meijder, A E F H* Histopathological, Enzyme histochemical and Electron microscopical Observations in Hypertrophic Obstructive Cardiomyopathy Path Microbiol *35* 81 85 1970

25 *Teare D* Asymmetrical Hypertrophy of the Heart in Young Adults Brit Heart J *10* 1 8, 1958

Acta path. microbiol scand Section A 81, 183-188, 1973

COLONY GROWTH IN PRIMARY CULTURES OF EHRLICH ASCITES TUMOUR USING THE AGAR METHOD FOR HUMAN BONE MARROW CULTURE

Soren Knudtzon

Department of Internal Medicine, Finsen Institute, Copenhagen, Denmark

A technique for colony formation in primary cultures of Ehrlich ascites tumour cells is described using the agar gel method of human bone marrow culture with human leukocytes serving as feeder cells The colony forming efficiency was low in the primary cultures but increased rapidly by recloning of colonies upon new feeder layers at 8 days interval Ascites tumours developed when single colonies were injected intraperitoneally into mice In contrary to human bone marrow cultures lymphocytes or leukemic cells in the feeder layer were able to stimulate cell proliferation of Ehrlich tumour cells Dialyzed human urine, active as a stimulator in mouse bone marrow cultures did not stimulate Ehrlich tumour cell cultures These findings suggests a difference, at least partly, in the nature of the feeder substance needed in the bone marrow cultures and the Ehrlich cell cultures HeLa cells, L cells and two established Ehrlich cell lines in culture formed colonies also when cultured by the agar method The soft agar technique offers a method for quantitative studies of drug sensitivity in primary cultures of Ehrlich ascites tumour cells and for the isolation of clones

A method for production of clones from HeLa cells in agar was originally described by *Puck et al* (1956) By this method, which is simple and rapid single HeLa cells grew into colonies, thereby allowing quantitative studies of cell growth, cell genetics and the response of cells to drugs and radiation

The technique has been applied with various modifications to a number of cell types such as virus transformed BHK 21 hamster ascites cells (*Sanders and Burford* 1964, *Macpherson and Montagnier* 1964), a P388D$_1$ mouse lymphoma cell line (*Pearson* 1964), L 1210 cells (*Himmelfarb et al* 1967), Hep 2 cells (*Macpherson and Montagnier* 1964), L-cells and FL cells (*Hausen et al* 1965) Colony growth in agar from murine haemopoietic cells has been reported (*Pluznik and Sachs* 1965, *Bradley and Metcalf* 1966) To obtain colony formation, either feeder cells, conditioned media from these cells or a serum or urine factor were necessary (*Bradley and Sumner* 1968, *Foster and Metcalf* 1968, *Robinson and Pike* 1970) Colony growth of human bone marrow cells has been described using either mouse kidney tubule cells (*Senn et al* 1967) or human leukocytes (*Robinson and Pike* 1970 a) as feeder cells

In this study colony growth of Ehrlich ascites tumour cells in primary cultures is re-

Received 5 x.72 Accepted 5 x 72

Request for reprints should be adressed to S Knudtzon, Finsen Institute, Department of Internal Medicine, Strandboulevard 49, 2100 Copenhagen, Denmark

ported, using the method described by *Robinson and Pike* (1970 a) for human bone marrow cultures with a feeder layer of human leukocytes

The method might be useful for quantitative studies of the chemotherapeutic response of Ehrlich ascites tumour cells in primary cultures and for the isolation of clones

MATERIALS AND METHODS

Mice and tumours A hypotetraploid strain of Ehrlich ascites tumour, obtained from Microbiological Associates, Inc, Bethesda, USA was carried by weekly serial passage in N/D mice (first generation hybrids of Swiss- and DBA mice) Passage was performed by intraperitoneal inoculations of 0 2 ml undiluted ascites fluid The cells were harvested with a Pasteur pipette under sterile conditions 6–8 days after inoculation and counted in a hemocytometer After counting, the cells were diluted in McCoy s 5 A medium to give the desired number of cells per plate in 10 µl

To compare the plating efficiency of a primary and of permanent cultures of Ehrlich ascites tumour cells, two established cell lines were obtained from the Fibiger Laboratory (Kongens Lyngby, Denmark) and examined by the agar technique The one, a near diploid cell line (ELD), was adapted to in vitro conditions in 1962, the other a near tetraploid cell line (ELT), in 1964 L cells and HeLa cells were obtained from stock lines that have been serially propagated at the Finsen Institute

L 1210 cells were obtained from Southern Research Institute, Birmingham, Alabama The tumour has been maintained in our laboratory since 1969 by weekly intraperitoneal inoculation into DBA mice

Nigrosin was used to estimate the cell viability (*Kaltenbach et al 1958*) Cells were only used for plating if the viability was over 95 per cent

Plating procedure Falcon petri dishes, 35 mm, were used for all experiments The medium was a modified preparation (*Robinson and Pike* 1970 a) of McCoy s 5 A medium containing 15 per cent fetal calf serum (Flow) This medium was used for convenience since the same medium was used for human bone marrow cultures in this laboratory McCoy s 5 A medium with fetal calf serum can be used equally well

The feeder layer was prepared by mixing medium at 37° C and previously boiled agar (Difco-Bacto-Agar), the final agar concentration being 0 5 per cent Human leukocytes were obtained from peripheral blood of healthy adults after two hours sedimentation at room temperature, and the cells were added to the agar medium to give a cell concentration of 10^6 per ml One ml of the mixture was poured per dish, and the agar allowed to harden at room temperature The feeder layer was used within 3–4 days, since the colony stimulating activity has been found to decrease after this One ml of a suspension containing 10^4 Ehrlich ascites tumour cells in medium and agar was plated on top of the feeder layer, the agar concentration being 0 3 per cent The dishes were incubated at 37° C in a humid incubator constantly flushed with 7 5 per cent CO_2 in air The cultures were followed by daily observation at 40 times magnification under a binocular dissecting microscope The number of colonies were counted after 7 days in incubation Only colonies containing more than 50 cells were counted The number of cells in a small colony was estimated by counting under the microscope The average number of cells per colony was calculated by counting the cells in a hemocytometer after resuspension of 50 pooled colonies in saline

For recloning experiments, colonies were removed with a fine Pasteur pipette, disrupted by pipetting in medium and the cell suspension replated on new feeder layers, 5×10^2 10^3 cells per plate

Conditioned medium was made by incubation in medium of peripheral leukocytes from a healthy adult or leukocytes from a patient with chronic granulocytic leukemia in blastic crisis the cell concentration being 10^7 per ml (*Chervenick and Boggs* 1970) After 9 days of incubation at 37° C in 7 5 per cent CO_2 in air, the supernatant was removed after centrifugation and filtered through a Millipore membrane (0 45 µ) 0 15 ml of the conditioned medium was used per plate instead of a feeder layer Conditioned medium from Ehrlich tumour cells was made by the same method except that the cell concentration was 10^6 per ml and the incubation time 3 5 and 8 days

A urine sample from at patient with chronic granulocytic leukemia was dialyzed using Visking tape against 2 liters of distilled water for 72 hours as described by *Robinson and Pike* (1970) After centrifugation the supernatant was filtered through a Millipore membrane (0 45 µ) and 0 1 ml added to each plate

filter that retains the granulocytes (*Kissmeyer Nielsen and Kjerbye* 1967)

Retransplantation studies with Ehrlich tumour cells were made by aspiration of a single colony into a syringe with 0 2 ml of Hanks' balanced salt solution followed by intraperitoneal injection into N/D mice

RESULTS

When Ehrlich ascites tumour cells were harvested from mice and plated upon a feeder layer of normal human leukocytes, colonies appeared in the upper agar layer Immediately after seeding the single cells were uniformly dispersed After 24 hours, cell clusters of 2–3 cells each were observed, and 72 hours after plating the cultures contained cell clusters of 10–20 cells On day 7 colonies were easy to count with an average of 5000 cells per colony the colonies being visible to the naked eye Almost all colonies formed were of similar size and had a round, compact structure The maximum colony size was obtained about day 14 the average number of cells per colony being 2×10^4 For routine use the colonies were counted on day 7 When tested 7 and 14 days after plating the viability of the cells in a colony was found to be >95 per cent and 10–20 per cent respectively measured on cells resuspended in saline

When varying numbers of Ehrlich cells were plated and the number of colonies counted 7 days later a linear relationship was found as shown in Fig 1

The colony forming efficiency (CFE number of colonies formed in per cent of number of cells plated) was low around 1 per cent, and was found to be independent of tumour cell age within 6–9 days after transplantation but dependent of a number of other factors

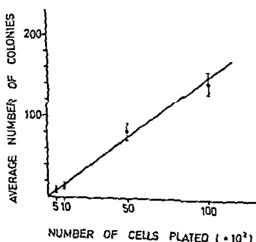


Fig 1 Relationship between number of Ehrlich cells plated and number of colonies counted 7 days later Each result represents the average of 3 plates ± 2 SD The slope is calculated from the sum of total observations

TABLE 1 *Relationship between Agar Concentration in the Upper Layer and Number of Colonies**

Agar concentration per cent	No of colonies/ 10^4 Ehrlich cells ± SD
0 1	55 ± 4
0 2	168 ± 7
0 3	180 ± 8
0 4	161 ± 7
0 5	110 ± 6

* Colonies counted 7 days after plating 10^4 Ehrlich cells per plate The results represents the average of 3 plates ± SD

In *Table 1* the number of colonies are listed when agar concentration of the upper *layer varied between 0 1 per cent and 0.5 per cent the optimal agar concentration being* 0 2–0 4 per cent

The CFE was found to be dependent of the number of cells *in the feeder layer* In most of the cultures no colonies were formed when the feeder layer was omitted and only 1–2 colonies were seen occasionally With increasing numbers of leukocytes and lymphocytes in the feeder layer increasing number of colonies were seen as shown in Fig 2 When Ehrlich tumour cells were plated on a feeder layer containing peripheral blood cells from patients with untreated acute myeloblastic leukemia the CFE was often increased in comparison with the CFE when normal leukocytes were used as feeder cells (Table 2) The plasma from the leukemic patients 0 15 ml per plate did not stimulate colony formation

Ehrlich tumour cells in the feeder layer 10^6 cells per plate did not stimulate colony formation and no colonies were seen when conditioned medium from Ehrlich cells or from peripheral leukocytes were used Dialyzed urine *from a patient with chronic granulocytic* leukemia did not stimulate colony formation The same urine was found active as

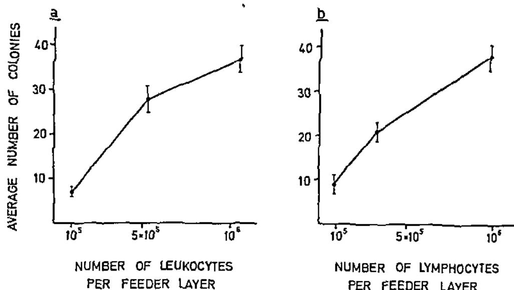


Fig 2 Relationship between number of colonies and number of leukocytes (a) and lymphocytes (b) in the feeder layer The percentage of lymphocytes in (a) and (b) were 27 and 100 respectively 10^4 Ehrlich cells in the upper layer Incubation time 7 days Each result represents the average of 4 plates ± SD

a stimulator of colony formation in mouse bone marrow cultures

By transferring resuspended Ehrlich tumour cells from 8 day-old colonies upon new feeder layers and repeating this at 8 day intervals the CFE gradually increased as shown in Table 3 The viability of the cells before each re plating was over 95 per cent

The CFE of the two established cells lines ELD and ELT was found to be 95 per cent and 15 per cent, respectively using the same method No colonies were formed when L 1210 ascites tumour cells were plated in primary cultures 10^4 cells per plate When HeLa and L cells were plated colonies were formed, the CFE being 37 per cent and 39 per cent, respectively (Table 3) When the feeder layer was omitted no colonies were formed

When single colonies from primary cultures of Ehrlich tumour cells were taken 10–15 days after plating and injected intraperitoneally into N/D mice 19 out of 20 mice developed ascites tumour and died 2–3 weeks after the injection

DISCUSSION

It has generally been found difficult to establish cell lines of mouse ascites tumour cells in monolayer culture and suspension culture

TABLE 2 *Ehrlich Cell Colony Formation Using Normal and Leukemic Blood Cells in the Feeder Layer**

Experiment number	Patient	Diagnosis	Myeloblasts in blood %	Number of colonies formed using leukemic feeder layer	Number of colonies formed using normal feeder layer
90	C A	AML	58	166 ± 6	56 ± 3
100	P B	AML	75	129 ± 7	140 ± 7
198	N I	AML	30	>500§	72 ± 5

* 10^4 Ehrlich cells per plate incubation time 7 days Results represents the average of 3 5 plates SD
§ Too numerous to count.

TABLE 3 *Colony Formation of Different Cells in Agar with Human Leukocytes as Feeder Cells*

Cell type	No of cells per plate	Average number of colonies per plate*	C F E %
Ehrlich in primary culture	10^4	180 (3)	1 8
Ehrlich replating 1	10^3	55 (3)	5 5
Ehrlich replating 2	5×10^2	197 (4)	39
E L D	10^2	95 (3)	95
E L T	10^2	15 (3)	15
HeLa	$5\ 55 \times 10^2$	203 (3)	37
L	10^2	39 (4)	39
L 1210 in primary culture	10^4	0 (3)	0

* No in parentheses indicate no of plates

some of the difficulties being a prolonged lag phase before cell division starts, and the poor recovery of most of the cultures initiated (*Guerin and Kitchen* 1960, *Guerin and Morgan* 1961)

By the method described, using leukocytes as feeder cells, primary cultures of Ehrlich ascites tumour cells are easily initiated without any significant lag period of the colony-forming cells The recloning experiments indicate that a cell line can be started and maintained with *no difficulties Quantitative* measurements can be made by counting the number of colonies, without the risk of losing cells by intervening medium changes

That a single cell initiates each colony is indicated by the linear relationship between the number of cells plated and the number of colonies formed and by the direct observation of the early cultures in the microscope In spite of this, the possibility remains that single cells surrounding a growing colony might become included in the colony during its growth

The plating efficiency is low in the primary cultures but the CFE is rapidly increased by recloning indicating an adaption of the cells to the in vitro milieu or a selection of certain cells with increased ability for growth in vitro That the CFE is dependent on intrinsic factors in the cell is indicated also from the difference in CFE between the two established cell lines ELT and ELD

Feeder cells or conditioned medium from these cells are often used to promote cell growth in vitro *Powell* (1958) found that explants of various tissues, macrophages and spleen monocytes from mice liberated a substance that prolonged the survival of Ehrlich tumour cells in culture *Puck et al* (1956) used irradiated non dividing HeLa cells as feeder cells for the production of clones from HeLa cells By modification of the procedures they found that the feeder cells could be omitted The need of a feeder layer to obtain cell growth in cultures probably indicates that the medium used and the procedures involved are not optimal

For the growth of murine haemopoietic cells in vitro using the soft agar technique, a feeder layer is needed, mouse kidney or mouse embryo cells being the most effective (*Pluznik and Sachs* 1965, *Bradley and Metcalf* 1966) Colony growth of human granulocytic progenitor cells using a feeder layer of mouse kidney cells has been reported, but human leukocytes *are more active as feeder cells* (*Robinson and Pike* 1970 a) Recent results from human bone marrow studies indicate that the neutrophil granulocyte is the source of the colony stimulating factor in the feeder layer (*Greenberg et al* 1971) and there is an indication that this factor may serve as a regulating factor of granulopoiesis in vivo (*Robinson and Pike* 1970)

The results reported in this study indicate that the colony stimulating factor, necessary for colony formation of Ehrlich tumour cells, is at least partly different from the factor needed to stimulate granulocytic colony formation when human and mouse bone marrow cells are plated In contrast to the human bone marrow studies using the same culture method normal lymphocytes and leukemic cells were able to stimulate cell proliferation in Ehrlich tumour cells The dialyzed urine with a known stimulatory effect upon mouse bone marrow did not stimulate colony formation of Ehrlich tumour cells

1971) and no single test was considered decisive. The removed specimens were immersed in liquid nitrogen, kept deepfrozen at a temperature of −70 C until cryosections for histochemistry, cut to 10 μ thickness were made at 23 C. The following enzyme determinations were made.

Alkaline and acid phosphatases were determined according to the methods of *Gomori (Pearse 1961)*, and adenosin triphosphatase (ATPase) and 5 nucleotidase by the methods of *Wachstein and Meisel* (1957). Non specific esterase was determined using the α naphtylacetate method of *Gomori* (1952), and leucine aminopeptidase (LAP) according to *Nachlas et al* (1957).

For estimation of lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and glutamate dehydrogenase (GluDH) activity, the methods outlined in *Pearse*'s (1961) manual were used.

Sections were also stained by haematoxylin and eosin, van Gieson, Giemsa and Gomori methods.

RESULTS

Alkaline Phosphatase

Distinct alkaline phosphatase activity was seen in the fibrous periphery of the sarcoid tubercles and in the lymphatic pulp located in the cytoplasmic rim of the lymphocytes around the epithelioid cell tubercles (Fig 1).



Fig 1 Staining for alkaline phosphatase activity in sarcoidosis. The epithelioid cells and giant cells in the tubercle show no activity, the fibrous periphery of the tubercle shows distinct activity. The cytoplasmic rim of the lymphocytes stains regularly. Magnification × 160

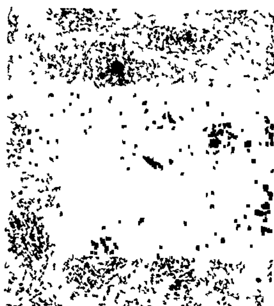


Fig 2 Staining for acid phosphatase in sarcoidosis. Marked activity is seen in the tubercles while activity in the intertubercular area is weak. Magnification × 40

The endothelial cells of the capillary wall also showed strong activity. The epithelioid and giant cell tubercles lacked any enzyme activity. All areas of degeneration were devoid of enzyme activity.

Acid Phosphatase

The pattern of this enzyme contrasted with that of alkaline phosphatase. Marked activity was seen in the tubercles in which particularly the nuclei of the epithelioid cells showed strong activity whereas staining in the plasma was weaker. The giant cells were sometimes black from lead precipitate and all nuclei stained strongly, obscuring the plasma partly. In the intertubercular area there was only weak activity in the form of fine granules in the cell cytoplasma. The fibrous tissue and the areas of degeneration showed no enzyme activity (Figs 2 & 3).

Adenosin Triphosphatase

Activity in the sarcoid tubercles was moderate. The density of staining in the epithelioid and giant cells was variable, appearing in

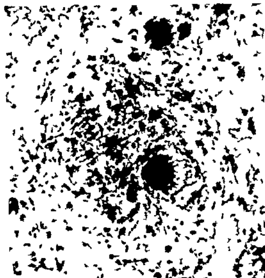


Fig 3 Acid phosphatase staining of a tubercle in sarcoidosis Strong activity is seen particularly in the nuclei of epithelioid cells and giant cells weaker staining in the cell plasma Magnification X 160

patches mainly in the peripheral areas However, a marked activity regularly appeared in the capillary walls in the intertubercular area (Fig 4)



Fig 4 Staining for adenosin triphosphatase in sarcoidosis Moderate activity is seen in the tubercles stronger activity appears in the capillary walls in the intertubercular area. Magnification X 160

5 nucleotidase

Distinct activity occurred in the form of coarse granules and larger clumps in the epithelioid cells and giant cells However, many cells lacked all activity and the same applied to the fibrous periphery of the tubercles. On account of positive demarcation lines, the general architecture occasionally had a lobulated appearance The demarcation lines often traversed the adjoining lymphatic pulp, which resulted in new tubercular borders much larger than the epithelioid cell tubercles as such In the lymphatic pulp the cytoplasmic rims showed strongly positive staining in some areas while activity was slight or nonexistent in other areas (Fig 5)

Non specific Esterase

Activity of non specific esterase was moderate in the nuclei of some epithelioid cells and giant cells, while the cytoplasm showed diffuse, weaker staining Some histiocyte nuclei outside the tubercles also showed strongly staining nuclei The lymphatic pulp generally



Fig 5 Staining for 5 nucleotidase in sarcoidosis Activity in the form of coarse granules is seen in the tubercles The lobulated appearance due to *positive demarcation lines traversing* the lymphatic pulp shows up The lymphatic pulp stains diffusely Magnification X 160

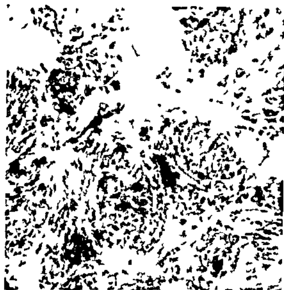


Fig 6 Staining for non specific esterase in sarcoidosis Moderate activity appears in the nuclei of epithelioid and giant cells but also as diffuse staining of the cytoplasm The lympatic pulp is mostly negative Magnification × 160



Fig 7 Staining for lactate dehydrogenase in sarcoidosis Strong activity in the epithelioid cells and giant cells is seen in the form of granules and as diffuse staining Lesser activity is also seen in the plasma of the lymphatic pulp Magnification × 160

was negative No activity was seen in the degenerative areas (Fig 6)

Leucinaminopeptidase

Weak to moderate activity was seen in less than half of the specimens, while the majority showed no activity Diffuse staining appeared in the cytoplasm of the epithelioid cells in some tubercles, the lymphatic pulp being always negative Fresh sections of skin were used as a standard reference

Lactate Dehydrogenase

There was strong activity in the cytoplasm of epithelioid cells and giant cells which occurred diffusely and might be in the form of small round granules Distinct though slightly lesser activity was seen in the plasma of the intertubercular cells In the areas with cellular degeneration there was still some enzyme activity whereas the areas of frank fibrinoid necrosis lacked all activity (Fig 7)

Malate Dehydrogenase

All areas containing fresh tubercles showed the same intense staining for MDH and LDH Against the blue background of diffuse cellular plasma there were numerous blue precipitate granules in the cytoplasm of epithelioid cells and particularly in that of the giant cells Enzyme activity in the intertubercular cell cytoplasm was also less marked here than in the tubercles Activity was absent from areas of frank degeneration but still occurred in areas showing degenerative cells

Glutamate Dehydrogenase

Staining for this enzyme was distinctly less intense than that for LDH and MDH Some activity appeared in the epithelioid cells in the tubercular centres and activity was clearly weaker in the tubercular periphery as well as in the intertubercular area

Control Sections

All control sections in sarcoidosis without substrate were negative

The normal lymph nodes showed the same staining for various enzymes as that described in the case of the lymphatic pulp in sarcoid lymph nodes For alkaline phosphatase capil

laries with strong activity were much more numerous than in sarcoidosis The activity of acid phosphatase and *non specific esterase*, seen in the epithelioid and giant cells in sarcoidosis, was mainly located to the histiocytes in the control nodes Activity of 5-nucleotidase was regularly moderate in the control nodes while adenosin triphosphatase activity was fairly strong in the control nodes because of their large numbers of capillaries In the case of oxidoreductases, the lymphatic pulp showed marked staining

COMMENTS

This enzyme study of sarcoid lymph nodes was limited to hydrolases and oxidoreductases Strong hydrolase activity was demonstrated for acid and alkaline phosphatase, moderate activity for ATP-ase, 5-nucleotidase and cholinesterase and weak unregular activity for leucinaminopeptidase

Distinct differences between various hydrolases appeared at the site of activity Non-specific alkaline phosphatase activity was present in the cytoplasm of the remaining lymphocytes and in the capillary endothelial walls In the tubercles, it was regularly demonstrable at the periphery only, in the cells resembling big fibroblasts, which formed the tubercular „capsule' Similar activity of alkaline phosphatase in the tubercular periphery has also been demonstrated by *Gusek* (1967)

Hydrolase activity in the epithelioid cells and giant cells was most remarkable in the case of acid phosphatase and was particularly strong in the nuclei There was no artifact nuclear staining since all control specimens were negative The moderate activity of adenosin triphosphatase, 5 nucleotidase and non specific esterase appeared more uniformly in the cytoplasm In some specimens, 5-nucleotidase activity showed specific patternforming borders while it followed the original tubercular configuration in others This intracellular activity of acid phosphatase, ATP-ase, 5 nucleotidase and non specific esterase fits well with the phagocytic capacity of the epithelioid and giant cells as postulated by *Williams et al.* (1969). *Lurie*'s finding (1964) that the amount of acid phosphatase is directly proportional to the host resistance against tubercle bacilli would fit in perfectly with the present consistent finding of strong acid phosphatase activity in the epithelioid and giant cells

Two of the oxidoreductases, LDH and MDH, showed strong activity whereas GluDH activity was distinctly weaker. This is in keeping with our earlier results (*Palva et al* 1972) concerning the quantitation of these enzymes in sarcoid tissue homogenates. The average activity figures in the homogenized sarcoid lymph nodes for LDH, MDH and GluDH were 44, 64 and 2 U/g per fresh tissue weight, respectively, *representing an* activity 400-2000 times as high as that in serum.

The role of the oxidoreductases is apparently of importance in the energy production and metabolism of epithelioid and giant cells LDH and GluDH play a part in the glycolysis cycle and MDH in the citric acid cycle, and the concentration of these enzymes in the cytoplasm of epithelioid cells and giant cells was more intense than the activity in the lymphocyte cytoplasm The increased activity of these enzymes has also been shown to enhance the host resistance against tubercle bacilli (*Lurie* 1964)

The aetiology of sarcoidosis is generally considered to be viral or tuberculous Recently, elevated titres—particularly for the herpes-like virus (HLV, EBV)—have been found to occur in sarcoidosis (*Hirshaut et al* 1970) This was also demonstrated by us (*Nikoskelainen et al* 1971) but the titres were similarly elevated in our control group of tuberculous patients Thus at the present time, no definite evidence of a viral aetiology is available

The histological picture, however, bears such strong resemblance to that of tuberculous infection (*Järvi et al* 1967, *Määttä* 1968) as to suggest that the sarcoidosis-type tissue response could represent the organism's overshooting reaction to infection with tubercle bacilli The main resistance mechanism

in the sarcoid lymph nodes might consist of a strong hydrolase activity, particularly in the form of acid phosphatase and of marked oxidoreductase activity The hyperactive epithelioid cells and giant cells might destroy tubercle bacilli by hydrolysing their phospholipid fraction so that bacilli can no longer be demonstrated by staining or culture, and very rarely in guinea pig inoculations (*Maatta* 1968) The immunological response mechanism, producing the proliferating sarcoid lymph nodes, remains unexplained for the present

REFERENCES

1 *Gomori, G* Microscopic Histochemistry University of Chicago Press, Chicago 207, 1952

2 *Gusek, W* Vergleichende Cytologie und Histogenese des Sarkoidosegranuloms Arch f klin u exper Derm *227* 24 53 1966 1967

3 *Hirshaut, Y, Glade, P, Vieira L O B D, Ainbender, E, Dvorak, B & Siltzbach, L E* Sarcoidosis, another disease associated with serologic evidence for herpes like virus infection The New Engl J Med *283* 502–506 1970

4 *Jarti, O H, Kalliomaki, J L, Maatta, K T, Palva, T K & Toivanen, A M* A bacteriological and histological study of hilar lymph nodes in a series of clinical sarcoidosis La Sarcoidose Rapports de la IVe Conference Internationale Paris 12–15 Septembre 1966 Masson & Cie 518–527 1967

5 *Lurie, M B* Resistance to tuberculosis Experimental studies in native and acquired defensive mechanisms Harvard University Press Cambridge Massachusetts 303 329 1964

6 *Mustakallio K K & Niemi M* Histochemie der Lysosomenenzyme des Sarkoidosegranuloms Der Einfluss von Prednisonbehandlung Arch f klin u exper Derm *227* 99–101 1966–1967

7 *Maatta, K T* Histological study of mediastinal lymph nodes in clinical sarcoidosis. A report of 86 cases Annales Academiae Scientiarum Fennicae, Series A, V Medica 138 Helsinki 1–106 1968

8 *Nachlas, M M, Crawford, D T & Seligman A M* The histochemical demonstration of leucine aminopeptidase J Histochem Cytochem *5* 264 278, 1957

9 *Nikoskelainen, J, Hannuksela, M & Palva, T* Fluorescent antibodies to EB (Epstein Barr) virus in sarcoidosis Scand J clin Lab Invest 27 Suppl *116* 49 1971

10 *Palva, T & Huhti, E* Mediastinoscopy in the evaluation of sarcoidosis In Jepsen, O & Sorensen, H R (Ed) Mediastinoscopy Proceedings of an International Symposium Odense University June 18–20, 1970 Odense University Press 60–66, 1971

11 *Palva, T, Raunio V & Vousiainen, R* Lactate malate and glutamate dehydrogenase activity in mediastinal lymph nodes in sarcoidosis Ann Clin Res. *4* 223–227, 1972

12 *Pearse, A G E* Histochemistry, J & A Churchill Ltd London 872 882, 911 912 1961

13 *Sutton, J S* Ultrastructural aspects of in vitro development of monocytes into macrophages epithelioid cells, and multinucleated giant cells In Second Decennial Review Conference on Cell Tissue and Organ Culture National Cancer Institute Monograph 26 Bethesda, Maryland 71–141 1967

14 *Wachstein M & Meisel E* Histochemistry of hepatic phosphatases of a physiologic pH with special reference to the demonstration of bile canaliculi Amer J Clin Path *27* 13–23, 1957

15 *Weiss, L P & Faucett D W* Cytochemical observations on chicken monocytes macrophages and giant cells in tissue culture J Histochem Cytochem *1* 47 65 1953

16 *Williams D Williams, W J & Williams, J E* Enzyme histochemistry of epithelioid cells in sarcoidosis and sarcoid like granulomas J Path *97* 705–709 1969

Acta path microbiol scand Section A *81*, 195–203, 1973

PRIMARY POLYCYTHAEMIA

3 Studies on the Significance of the History of the Disease and of the Treatment for the Development of Clones in Bone Marrow Cells

JAKOB VISFELDT, SIXTEN FRANZÉN, ARNE NIELSEN and BERNHARD TRIBUKAIT

Institute of Pathology, Frederiksberg Hospital, Copenhagen,
Radiumhemmet, Karolinska Sjukhuset, Stockholm,
Department of Statistics Institute of Human Genetics, Copenhagen,
Radiobiologiska Institutionen, Karolinska *Institutet, Stockholm*

The study comprises 75 patients with primary polycythaemia treated at the Radiumhemmet in Stockholm The patients were selected, in that a reasonable representation of four groups were *aimed at untreated, treated without myelofibrosis, treated with myelofibrosis*, and treated patients in whom incipient transition into leucaemic phase was suspected clinically All the treated patients had received ^{32}P and/or Myleran Chromosome analyses of blood and bone marrow were made once or several times Twenty seven *clones of bone marrow* cells with abnormal chromosomal pattern were demonstrated in 26 patients Twenty of the clones comprised more than 50 per cent of the cells analysed It is especially emphasized that the F deletion previously described was demonstrated also in megakaryocytes in mitosis and, consequently, it must be supposed that the F deletion can be induced in primitive, multipotent haemopoietic cells In order to clarify the influence of ^{32}P therapy on the genesis of the clones studies into the relationship between some clinical parameters and the clone formations were made The survey shows that the clones occur almost exclusively in patients treated with ^{32}P, that in our series the clones were not found in patients treated exclusively with Myleran, and that clones might appear in very few untreated patients. As regards the three groups of treated patients, it applies that generally the clones do not occur until the calculated dose *per year is appreciable* (> 2 5 mCi), *and that the clones are found* almost exclusively in patients treated over prolonged periods (> 2 years) with ^{32}P

In previous publications concerning our series of patients, we have reported some histological and cytogenetic features (8, 9, 10)

More patients have now been included, and the present series comprises 75 cases The observation period is longer in many of the patients, and it has been possible to repeat the chromosome analyses in some cases

Received 18 x 72 Accepted 10 xi 72

Requests for reprints should be addressed to Jakob Visfeldt, Institute of Pathology, Frederiksberg Hospital DK 2000 Copenhagen F, Denmark

13*

The objects of the present report are

1) a description of the enlarged group and of the new clinical, histological and cytogenetic details which were revealed,
2) study of the relationship between some clinical parameters and the clone formations

MATERIAL AND METHODS

The material comprises 75 patients from the Radiumhemmet in Stockholm All 75 patients were evaluated clinically and treated by one of us

(S F) The patients were selected, because we have insufficient capacity for including all cases Further more, the aim was to have a reasonable representation of four groups untreated, treated without myelofibrosis, treated with myelofibrosis, and treat ed patients in whom incipient transition into a leucaemic phase was suspected clinically All treated patients had received ^{32}P and/or Myleran, in a few cases this therapy had been supplemented by other chemotherapeutics or very limited external irradiation

In connection with routine examination and treatment of the patients, histological and cyto logical examination of the bone marrow was made and, at the same time, bone marrow and peri pheral blood were obtained for chromosome ana lysis

The aim was to analyse between 50 and 100 cells from both blood and bone marrow, from each patient In a few patients, not more than between 10 and 20 analysable cells were found in the bone marrow aspirate If less than 10 cells from the bone marrow were analysed, the patient concerned was excluded from the series In some patients the analyses were repeated

The bone marrow specimens for chromosome analysis were prepared using a modification of the direct method of *Tjio & Whang* (7) The blood samples were analysed after 48 hours culture and in other respects by the method of *Moorhead et al* (6)

Complete chromosome analyses were made of each individual cell and many of the cells were photographed

The clinical parameters to which the present paper will be specially devoted, are *length of history before treatment* The clinicist (S F) evaluated the onset of the disease on the basis of symptoms and signs, which in all probability could be ascribed to the patient s polycythaemia vera The number of years from onset of the disease and until the institution of treatment with ^{32}P or Myleran was ascertained Furthermore, *period of treatment with* ^{32}*P*, comprising the number of years between the first and the last ^{32}P treatment, prior to the first chromosome analysis

Total ^{32}*P dose before chromosome analysis* is assessed as the total oral dose measured in mCi

Period of treatment with ^{32}P and total ^{32}P dose before chromosome analysis are included in the fictive term *calculated dose per year* This term is employed only as a first approximation to the risk of clone formation incident to irradiation It has no bearing on the therapeutic single dose If only one single dose is given the period of treatment is estimated at one year

RESULTS

In the bone marrow specimens from the 75 patients, large clone formations were found *in 26 patients*, one patient had two completely different clones and consequently, the total number of clones is 27

Table 1 presents the distribution of clones occurring in the various groups of patients

Table 2 shows a number of data concerning the clones, including the size of the clones and their karyotypes

TABLE 1 *Patients with Primary Polycythaemia at Various Stages Carrying Clones with Abnormal Karyotypes in Bone Marrow*

No of Patients	No of clones
17 untreated	2 (z o)
36 treated, without myelofibrosis	11 (f i,j,l,m + v n o x,y,æ)
14 treated with myelofibrosis	9 (e g h k,q r s t u)
8 treated, leucaemic phase suspected	5 (a b,c d p)

DISCUSSION

Cytogenetic Investigations

The reader is referred to cytogenetic investigations reported in our previous publications (8, 9, 10) In the present report only few recent data and observations will be discussed

Number and size of clones As stated in Table 1, 27 clones were found in 26 patients In all cases the clones were fairly large, in 7 cases comprising between 25 per cent and 50 per cent of the bone marrow cells analysed, in 20 cases more than 50 per cent

In one patient, two well defined clones were found in the bone marrow At the examination in 1969, one clone (m) was revealed comprising 93 per cent of the bone marrow cells at the re-examination in 1971, the clone m comprised 36 per cent only and, at the same time, a new clone (v) of a completely different karyotype had developed and comprised 53 per cent There were *no simi-*

TABLE 2 *Clones with Abnormal Chromosome Pattern in Bone Marrow from Patients with Primary Polycythaemia*

Clone	♂/♀	Age	Date of analysis	No of cells analysed	% clone cells	Karyotype
a	♂	80	Jan 69	51	56	46,XY,B–,16+
b	♂	62	Jan 69	60	73	46,XY,F?
			March 69	28	100	
c	♀	64	March 69	49	94	46,XX,1?+,B–,C–,C–,16+,16+,16+
d	♀	83	May 69	23	83	46,XX,2–,3+,Bq+
e	♀	81	June 69	51	32	46,XX,F?–
f	♂	58	June 4th 69	12 }	35	47,XY,C+
			June 25th 69	63 }		
			March 71	53	36	
g	♀	64	June 69	26	61	46,XX F?–
h	♀	78	June 69	12	58	46,XX,C–,D+,Dq–,F?–
i	♀	73	Oct 69	100	91	46,XX,2+,C–
j	♂	60	Oct 69	53	87	46,XY,F?–
k	♂	72	Oct 69	79	100	46,XY,Dq–
l	♂	73	Oct 69	100	58	46,XY,B+,C–,C–,D+
			Nov 70	40	80	
m	♀	78	Nov 69	92	93	46,XX,1?+
m }			Feb 71	53	{ 36	46,XX,1?+
v }					{ 53	44,XX,2q–,3–,Bq+,E–
n	♀	62	Feb 70	30	63	46,XX,C–,D+,Gs+
			May 71	33	70	
o	♀	70	Feb 70	53	25	46,XX,Dq ,Ep+
p	♂	60	Feb 70	23	91	45,XY,3–,Cr
			April 70	53	85	
q	♂	40	March 70	67	84	46,XY,F?–
r	♂	73	May 70	12	42	46,XY,F?–
s	♂	67	March 70	24	54	47,XY,C+,F?–
t	♀	59	May 70	26	31	46,XX,E–,G+
u	♀	73	May 70	15	40	46,XX Gq–
x	♀	72	Jan 71	50	82	47,XX,C+
y	♀	78	Sep 71	43	88	46,XX,Dq–
z	♂	81	Dec. 71	50	76	47,XY,C+
æ	♂	55	March 72	17	94	44,XY,B–,C–,Eq+
ø	♂	72	April 72	20	45	46,XY,F?–

larities in the two clones which would make it probable that a development from one clone to the other had taken place (Figs 1 and 2)

F-deletion It appears from Table 2 that 7 of the 27 clones were characterized by an F-deletion alone The specificity of the F-deletion in primary polycythaemia has been discussed in a number of reports from The Royal Marsden Hospital (1, 2, 4, 5) and by us (9, 10)

The F-deletion was nearly always found in patients who had received ³²P therapy However, *Millard* (3) reported this F deletion also in a patient who had been treated exclusively by venesection We have now found the F-deletion also in an untreated patient and, consequently, we are able to confirm that this aberration *may* occur in patients with primary polycythaemia who have not received radiation treatment or chemotherapeutics

Furthermore, it should be pointed out that we have seen quite definitely the F-deletion in megakaryocytes in mitoses This is an interesting finding, because now it must be sup-

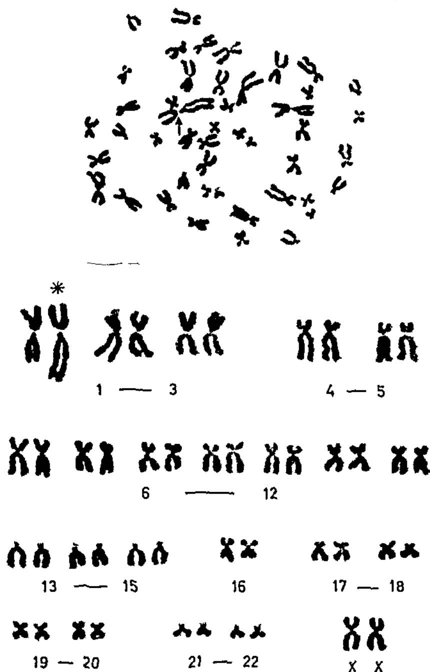
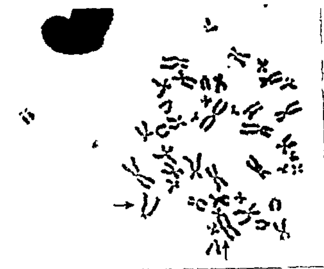
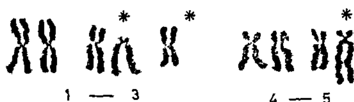
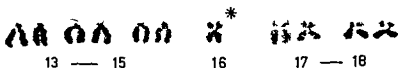


Fig 1 Clone m Karyotype of clone cell from bone marrow from a ^{32}P treated patient with primary polycythaemia 46 chromosomes translocation of material to a chromosome No 1 The clone comprised 93 per cent of analysed cells Nov 1969 Cf Fig 2









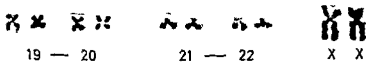


Fig 2 Clone v New clone from repeated bone marrow examination on the patient discussed in Fig 1 Karyotype with 44 chromosomes, translocation from a chromosome No 2 to a chromosome in the 4/5 group, missing chromosomes No 3 and No 16 Clone v comprised 53 per cent and clone m 36 per cent of analysed cells Feb 1971

posed that the F-deletion can be induced in primitive, multipotent haemopoietic cells

Our series includes 17 untreated patients, and clone formations have now been found in two cases Apart from the patient with the F-deletion, one patient presenting a clone with an extra group C chromosome, was found

Aneuploidy Lawler et al (2) discussed aneuploidy in patients with primary polycythaemia and ventilate that it might be an inherent tendency towards non disjunction in this disease This hypothesis can easily explain what we experienced in our series that in many patients with primary polycythaemia there is a higher incidence of non modal cells than in corresponding age groups of the normal population

History of Disease and Development of Clones

Length of history before treatment This period is defined as stated under "Material and Methods", and is evaluated for the four groups, which are again classified according to occurrence of clones, so that we work with a total of 8 groups

In Fig 3, number of patients in "the group" with a history shorter than a given number of years, is plotted against the corresponding number in "the other groups" This is done for each of the years, which are taken into consideration (1–21 years)

If the two distributions compared hereby, are uniform, the points will lie on a straight line Three groups present a picture which indicates a straight line

The group "treated, + myelofibrosis, + clones", comprising 9 patients has a shorter history before treatment than all other groups The group "treated, + leucaemia, – clones", comprising 3 patients, has a long history before treatment The group "treated, + leucaemia, + clones", comprising 5 patients, has a short history before treatment

This is in agreement with the fact that *both as regards patients who develop myelofibrosis, and patients who enter into a leucaemic phase, there is a greater tendency for clones to form when the disease develops rapidly into the stage where treatment becomes necessary*

Period of treatment with ^{32}P, total ^{32}P dose before chromosome analysis, and calculated dose per year For more detailed analysis of the radio biological effect of the radioactive phosphorus given, both the total dose and the time factor must be taken into account

Fig 4 presents the relationship between period of treatment and calculated dose per year for all patients, classified into untreated treated without myelofibrosis, treated with myelofibrosis and treated with leucaemia, and whether or not clones have been found in the bone marrow The total ^{32}P dose can be read directly as the product of the parameters recorded

A characteristic feature of the figures is that, as long as the *calculated dose per year* is low (defined as less than 2 5 mCi), only few of the patients have developed clones There are 5 patients, and in only one was a clone found, and this patient has been treated with ^{32}P for a period as long as 20 years Only one patient has been treated with ^{32}P for a longer period, i e, 23 years Only two of the 17 patients who received no treatment at all, developed clones, and only one of the 15 patients who had received therapy other than ^{32}P, developed clones This patient however had been treated with splenic irradiation Thirteen of the 15 patients had received no other treatment than Myleran Hence, clone formations were not found in any of the patients who had been given Myleran only

Furthermore, it is characteristic of the figures that if the *period of treatment* with ^{32}P was short (defined as 2 years or less), clones had not developed Eight of the patients were treated with ^{32}P for 2 years or less, and only one of these patients developed a clone, i e, a patient with myelofibrosis This patient, however, had received 16 mCi during the two years, which is a high dose in such a short time The finding of clones in

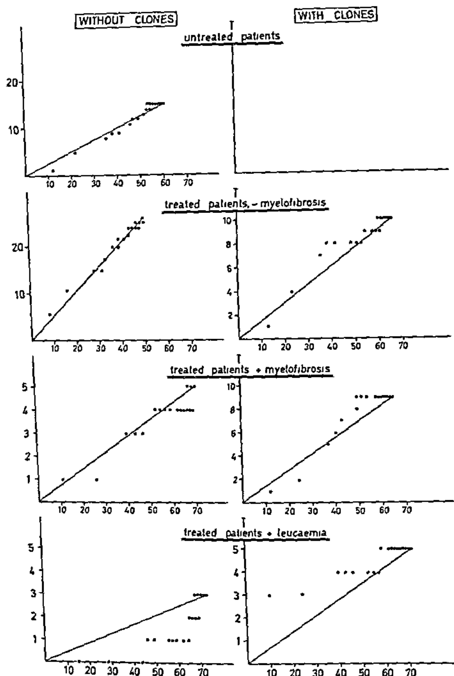


Fig 3 Distribution of length of history before treatment in single groups compared with distribution in the other groups Ordinate accumulated number in single group Abscissa accumulated number in other groups

the various groups, in relation to the period of treatment, is shown in Table 3 In all three treated groups, there is a significantly higher number of cases with clones in patients treated with ³²P for more than 2 years, as compared with all the patients treated for 2 years or less (one sided hypergeometric test without fibrosis $P < 0.5$ per cent, with

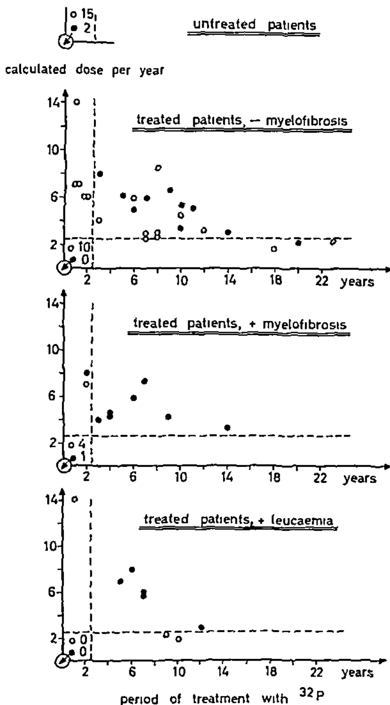


Fig 4 Relationship between period of treatment and calculated dose per year ○ patients without clones. ● patients with clones

fibrosis P < 0.5 per cent, with leucaemia P < 0.5 per cent)

Hence, it can be concluded that *the clones occur almost exclusively in patients treated with ^{32}P*, and that, *in our series, no clones were found in patients treated with Myleran only*. Furthermore it applies to all three of the treated groups that *the clones usually will*

TABLE 3 *Number of Clones in Different Groups According to Period of Treatment*

	No treatment with ^{32}P		Treatment with ^{32}P for 2 years or less		Total		Treatment with ^{32}P for more than 2 years	
	No	Of which with clones	No	Of which with clones	No	Of which with clones	No	Of which with clones
Untreated	17	2		•	17	2	•	•
Patients without *myelofibrosis*	10	0	5	0	15	0	21	10
Patients with *myelofibrosis*	5	1	2	1	7	2	7	7
Patients with leucaemia	0	0	1	0	1	0	7	5
Total	32	3	8	1	40	4		

not develop until the calculated dose per year is appreciable (> 2 5 mCi), and that *the clones were found almost exclusively in patients who had received* ^{32}P *over a long period* (> 2 years)

This work has been supported by grants from the *Swedish Riksforeningen mot Cancer*, and from the *World Health Organization*

REFERENCES

1 *Kay, H E M, Lawler, S D & Millard, R E* The chromosomes in polycythaemia vera. Brit J Haemat *12* 507–527, 1966

2 *Lawler, S D, Millard, R E & Kay, H E M* Further cytogenetical investigations in polycythaemia vera Europ J Cancer *6* 223–233, 1970

3 *Millard, R E* Personal communication, 1970

4 *Millard, R E, Kay, H E M & Lawler, S D* Chromosome studies in patients with polycythaemia vera after treatment with ^{32}P Radiation Induced Cancer, IAEA, Vienna, 1969 pp 289–303

5 *Millard, R E, Lawler, S D, Kay, H E M & Cameron, C B* Further observations on patients with a chromosomal abnormality associated with polycythaemia vera Brit J Haemat. *14* 363–374, 1968

6 *Moorhead, P S, Nowell, P C, Mellman, W J, Battips, D M & Hungerford, D A* Chromosome preparations of leucocytes cultured from human peripheral blood Exp Cell Res *20* 613–616, 1960

7 *Tjio, J H & Whang, J* Chromosome preparations of bone marrow cells without prior *in vitro* culture or *in vivo* colchicine administration Stain Technol *37* 17–20, 1962

8 *Visfeldt, J* Limitations of chromosome analysis as a method for biological dosimetry of chronic external radiation and of internal exposure to therapeutic radiophosphorus Acta path microbiol scand Sect A *79* 233–242, 1971

9 *Visfeldt, J* Primary polycythaemia. 2 Types of chromosome aberrations in 21 clones found in bone marrow samples from 50 patients Acta path microbiol scand Sect A *79* 513–523 1971

10 *Visfeldt J, Franzen, S & Tribukait, B* Primary polycythaemia. 1 Correlations between the histologic appearances and the chromosome pattern of the bone marrow cells during the disease Acta radiol (Ther Phys Biol) *10* 86–114, 1971

Acta path microbiol scand Section A 81, 204–212, 1973

PITUITARY WEIGHT AND THE HISTOLOGY OF THE PROSTATE IN ELDERLY MEN

An Analysis in an Autopsy Series

OLAV A HAUGEN

Ullevål Hospital, Department of Pathology, University of Oslo, Oslo, Norway
Head Kristen Arnesen M D

Pituitary weight variation was studied in a consecutive autopsy series of 170 men over 40 years of age, among whom none had clinically manifest prostatic carcinoma or had previously been subjected to prostatic surgery Among several factors tested by multiple regression analysis, a statistically significant and positive correlation between pituitary weight and body length only was demonstrated Patients with a histologically normal prostate showed a significant decrease of pituitary weight with advancing age, the weight being maintained to a larger extent in patients with benign hyperplasia and neoplasia of the prostate In the 7th decade of life, patients with benign hyperplasia and carcinoma accompanied by hyperplasia showed significantly higher pituitary weights than normal controls The results from the multiple regression analysis indicated that pituitary weight as such cannot be used to discriminate between various forms of prostatic histology

Some form of endocrine derangement in the ageing male has been postulated as a possible explanation of the high frequency of benign hyperplasia and carcinoma of the prostate in elderly men (*Lower* 1933, *Moore* 1947, *Sommers* 1957) However no conclusive evidence in support of this hypothesis has been given

Huggins & *Russell* (1946) demonstrated that atrophy of the prostate in dogs was more pronounced after hypophysectomy than after castration and suggested that pituitary hormones might have some direct effect upon the prostate In rats a synergistic effect between some pituitary hormones and testosterone on the prostate has been observed (*Lostroh* & *Li* 1957, *Van der Laan* 1960, *Grayhack* 1963) and it has been claimed that the prostate may be one of the target organs of prolactin (*Asano* 1965, *Asano et al* 1971)

Received 1 xi 72 Accepted 1 xi 72

Requests for reprints should be addressed to *Department of Pathology Ullevål Hospital Oslo 1*

As a part of an investigation of the relationship between pathological growth of the prostate and the morphology of certain endocrine organs, the present report describes the findings of pituitary weights in an autopsy series

Pituitary weight was analysed in relation to prostatic histology, as previously reported (*Harbitz* & *Haugen* 1972), and to several other factors which *a priori* were assumed to influence pituitary weight The effect of these factors has been assessed by single variable analysis and by multiple regression analysis, which facilitates a simultaneous study of several factors and a ranking of their relative importance

MATERIAL AND METHODS

The pituitary glands and the prostates from 206 consecutive autopsies of men over 40 years of age were collected during a 3 month period 1967 1968 Thirty six patients were excluded from the ana

lysis for the following reasons Previous prostatic surgery (24 patients), oestrogen treatment for clinically manifest prostatic carcinoma (six patients), secondary tumour invasion of the prostate or testes (four patients), and seminoma of the testis (one patient) In one instance the pituitary weight was not recorded The analysis was finally based on 170 patients among whom none had clinical evidence of prostatic carcinoma

The pituitary gland was removed together with a bone block surrounding the sella turcica, as described by *Mellgren* (1945) The diaphragm was cut open and the whole specimen fixed in Helly s fluid (Zenker Formol solution) for 24 hours, The fixed gland was lifted out and the dura thoroughly removed Excess of fixative was blotted off with filter paper and the stalk removed The *total pituitary weight* was recorded to the nearest milligram on a Mettler H 5 balance

The procedures of dissection and histological examination of the prostate have been described previously (*Harbitz & Haugen* 1972) The presence of benign nodular hyperplasia (BNH), carcinoma (C), atypical glandular proliferation (AGP) or diffuse atrophy (DA) of each prostate was noted The presence of AGP in prostates showing carcinoma was not recorded A histologically normal prostate (N) showed none of the characteristics mentioned above

The histological findings in the prostates of 170 patients analysed in the present series appears from Table 1

Clinical Data

Clinical data were recorded on the basis of the clinical notes and prepared for the computer analysis

Statistical Methods

Modified Student s t tests accounting for unequal variances and numbers of individuals were used for testing differences between arithmetic means and for testing differences between slopes of regression lines (*Snedecor & Cochran* 1967) n_A and n_B being the number of observations in the groups to be compared, p values were based on the least of n_A 1 and n_B 1 (for means) and n_A 2 and n_B 2 (for slopes) degrees of fredom. P values below 0 05 were regarded statistically significant.

Corrections for differences in age and body length were calculated according to the indirect method of standardization (*Armitage* 1971), using the age or body length specific pituitary weights in the total series as standard weights

Multiple regression analysis was applied as previously described (*Haugen & Harbitz* 1972), using pituitary weight (X_1) as the *dependent* variable The following factors either bivariate (1 0, labelled X_2 to X_{12}) or continuous (labelled X_{13} to X_{15}), were treated as *explanatory* (independent) variables

Histology of the Prostate

X_2 Benign nodular hyperplasia (BNH)
X_3 Atypical glandular proliferation (AGP)
X_4 Carcinoma (C)
X_5 Diffuse atrophy (DA)

Cause of Death

X_6 Cardiovascular disease*
X Malignant tumour

Duration of Final Illness

X_8 1–7 days
X_9 > 7 days

Other

X_{10} Steroid hormone treatment§
X_{11} Diabetes mellitus
X_{12} Liver cirrhosis
X_{13} Age
X_{14} Body weight
X_{15} Body length

Initially, forward stepwise regression analysis was run until all explanatory variables which were partially significant at the 5 per cent level at each step were included Thereafter, the selected variables, together with all groups of prostatic histology (X_2–X_5), were included in the full multiple regression analysis Regression coefficients were calculated according to the method of least squares Differences between regression coefficients for the various groups of prostatic histology were tested by an F test (*Scheffe* 1959)

The analysis was based on a standard program for multiple regression analysis (NRSR) developed at The Norwegian Computing Center, Oslo, and was conducted on a Univac 1108 computer

RESULTS

The pituitary weights showed an approximately normal distribution (Fig 1) with a

---

* includes death from myocardial infarction (49 cases), cerebrovascular and peripheral vascular disease (15 + 7 cases), rheumatic valvular disease (4 cases), miscellaneous cardiovascular disorders (11 cases)

§ includes treatment with corticosteroids (7 cases), anabolic steroids (nortestosterone) (5 cases), or both (11 cases)

TABLE 1 *Histological Diagnoses of the Prostate in 170 Patients*

Age	N	DA	BNH	C+BNH	C	AGP+BNH	AGP
40–49	1	1	2	0	0	0	0
50–59	10	5	10	3	1	5	0
60–69	7	1	22	15	5	6	1
70 79	0	1	28	21	0	5	1
80 +	0	0	8	9	0	2	0
Total	18	8	70	48	6	18	2

N = normal histology, DA = diffuse atrophy, BNH = benign nodular hyperplasia, C – carcinoma, AGP = atypical glandular proliferation

mean value of 667 2 mg, the median being 648 mg

Pituitary weight in relation to age is presented in Table 2 The number of patients below 50 years of age was small, and the low pituitary weights observed in these patients may have occurred by chance The highest mean weight was observed in the 6th decade of life while a slight decrease was seen in subsequent decades The high mean weight in patients beyond the age of 80 years was markedly influenced by a single extreme observation if this outlier be disregarded, the mean weight will come down to 628 8 mg The correlation between pituitary weight and age was negligible (Table 8)

The pituitary weight showed a slight trend to increase with increasing body weight (Table 3), but the relation to body weight was also weak, and the correlation coefficient was not statistically significant ($p > 0\ 05$) (Table 8)

The mean pituitary weight increased with increasing body length (Table 4) The association between pituitary weight and body

TABLE 2 *Pituitary Weight (mg) in Relation to Age*

Age	No patients	Pituitary Weight Mean	S D	Range
40–49	4	605 3	45 4	538–636
50–59	34	702 0	146 0	388–998
60–69	57	657 0	110 9	475–902
70–79	56	657 6	118 3	394–950
80 +	19	677 6	257 5	408–1557
All	170	667 2	142 4	388–1557

S.D Standard deviation

TABLE 3 *Pituitary Weight (mg) in Relation to Body Weight (kg)*

Body weight	No patients	Pituitary weight Mean	S D
< 50	25	622 2	132 0
50–59	45	660 5	132 9
60–69	44	670 2	188 5
70–79	36	704 9	114 7
80–89	14	647 1	82 5
90 +	6	704 8	88 9
All	170	667 2	142 4

S D Standard deviation

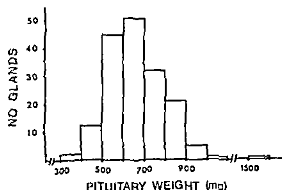


Fig 1 Distribution according to pituitary weight in 170 men over 40 years of age

TABLE 4 *Pituitary Weight (mg) in Relation to Body Length (cm)*

Body length	No patients	Pituitary weight Mean	S D
< 160	4	566.2	57.7
160–164	17	636.8	132.3
165–169	35	636.2	99.1
170–174	54	651.3	127.6
175–179	40	714.6	190.7
180–184	13	686.7	123.7
185 +	7	770.1	106.4
All	170	667.2	142.4

S D Standard deviation

length was not particularly strong, but the correlation coefficient was highly significant ($p < 0.001$) (Table 8)

Cause of death, duration of final illness, treatment with steroid hormones other than oestrogens, or the presence of liver cirrhosis did not obviously influence pituitary weight (Table 5)

Patients with diabetes mellitus showed high pituitary weight, although the difference between the crude mean pituitary weights of *diabetics and non diabetics was not statistically* significant ($p > 0.10$) The pituitary weight in diabetics also remained high if

TABLE 5 *Pituitary Weight (mg) in Relation to Cause of Death Duration of Final Illness Steroid Hormone Treatment Diabetes Mellitus and Liver Cirrhosis*

	No patients	Observed mean	S D	Age adjusted mean
Cause of death				
Cardiovascular disease	86	665.6	145.3	667.8
Malignant tumour	44	665.5	148.1	666.8
Other conditions	40	673.1	133.5	666.9
Duration of final illness				
< 1 day	37	670.1	102.3	670.1
1 7 days	34	642.5	106.0	646.4
> 7 days	99	675.3	164.8	683.3
Steroid hormone treatment	23	657.2	153.8	660.8
Diabetes mellitus	7	749.0	119.2	746.4
Liver cirrhosis	5	675.2	184.8	667.3
All	170	667.2	142.4	

S D Standard deviation

TABLE 6 *Mean Pituitary Weight (mg) in Relation to Histology of the Prostate* and Age*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40 49	(636.0	(628.0)	578.5				
50–59	699.1	655.8	705.4	710.0	(800.0)	722.6	
60 69	579.3	(729.0)	673.3	687.8	703.8	589.5	(513.0)
70 79		(550.0)	670.5	637.3		684.0	(697.0)
80 +		–	668.1	687.1		673.0	
All	649.0	648.2	672.9	667.0	719.8	662.0	605.0
S D	87.3	206.1	117.4	189.0	110.4	123.4	91.0

* For abbreviations and numbers see Table 1
Figures in brackets refer to single observations

S D Standard deviation

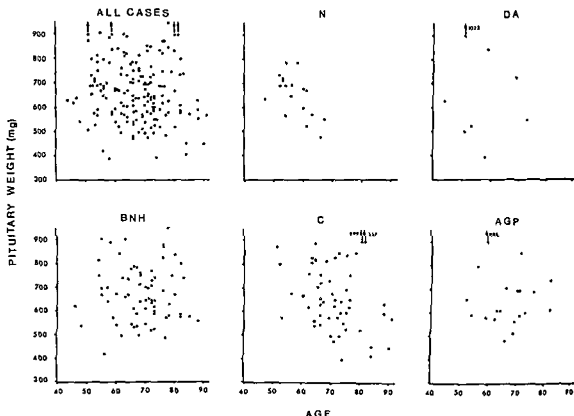


Fig 2 Scatter diagrams showing pituitary weight in relation to age in all 170 cases, and in men with histologically normal prostate (N), diffuse atrophy (DA), benign nodular hyperplasia (BNH), carcinoma (C), and atypical glandular proliferation (AGP) of the prostate ○ indicates cases in which BNH was not present

corrections were made for differences in age (Table 5) and body length (735 + mg)

As shown in Fig 2, patients with a histologically normal prostate showed a substantial reduction of pituitary weight with age In patients with BNH, C + BNH, and AGP + BNH no definite trend emerged from the scatter diagram Within all histological groups, however, and at all age levels there was a wide variation in pituitary weights The

TABLE 7 *Pituitary Weight (mg) in Relation to Histology of the Prostate Correction for Differences in Age and Body Length*

Histology of the prostate	No patients	Observed mean	Corrected for age	Corrected for body length
N	18	649 0	637 7	636 6
DA	8	648 2	637 2	649 4
BNH	70	672 9	674 8	679 4
C + BNH	48	667 0	670 3	661 2
C	6	719 8	722 8	697 4
AGP + BNH	18	662 0	657 4	664 8
AGP	2	605 0	667 3	626 1

* For abbreviations and numbers, see Table 1

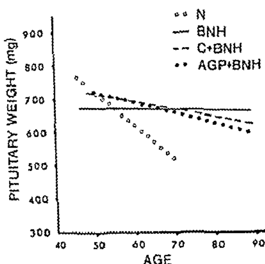


Fig 3 Regression lines for pituitary weight on age in different groups of prostatic histology Normal histology (N) benign nodular hyperplasia (BNH) carcinoma with BNH (C + BNH) atypical glandular proliferation (AGP) with BNH (AGP + BNH)

regression lines for pituitary weight on age in patients with N, BNH, C + BNH, and AGP + BNH of the prostate appear from Fig 3 The differences in slopes of the regression lines were not statistically significant ($p > 0.05$), but the regression coefficient for N differed significantly from zero ($p < 0.002$)

The lowest mean pituitary weight was seen in patients in whom the prostate histologically remained normal in the 7th decade of life (N) (Table 6). Low pituitary weight was also observed in patients with DA In patients with BNH, C + BNH and AGP + BNH, the pituitary weight was maintained with increasing age In the age group 60–69 years, patients with BNH and C + BNH had significantly higher pituitary weights than patients with normal prostate ($p < 0.05$) The few patients with C alone also showed high pituitary weight, but the latter was not significantly different from that observed in normal con-

TABLE 8 *Relationship between Pituitary Weight and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variable	X_1 Pituitary weight (n_1 = 172) Coefficient of correlation	Significant at level
*Histology of the prostate**		
X_2 BNH (n = 136)	0 030	0 697
X_3 AGP (n = 18)	-0 028	0 715
X_4 C (n = 54)	0 027	0 729
X_5 DA (n = 8)	-0 030	0 700
Cause of death		
X_6 Cardiovascular disease (n = 86)	-0 008	0 919
X_7 Malignant tumour (n = 44)	-0 007	0 924
Duration of final illness		
X_8 1 7 days (n = 34)	-0 093	0 229
X_9 > 7 days (n = 99)	0 066	0 389
Other		
X_{10} Steroid hormone treatment (n = 23)	0 028	0 717
X_{11} Diabetes mellitus (n = 7)	0 119	0 122
X_{12} Liver cirrhosis (n = 5)	0 010	0 900
X_{13} Age (n = 170)	-0 058	0 452
X_{14} Body weight (n = 170)	0 141	0 067
X_{15} Body length (n = 170)	0 251	0 001

* For abbreviations see Table 1
n_1 Number of cases in which pituitary weight was recorded
n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continuous variables)

TABLE 9 *Pituitary Weight and Histology of the Prostate* Full Regression Analysis*

Explanatory variables	X_1 Pituitary weight ($n_1 = 170$) Partial correlation coefficient	Partial regression coefficient	Significant at level
X_{15} – Body length (n = 170)	0.259	5.70	0.001
X_2 – BNH (n = 136)	0.077	30.65	0.322
X_3 – AGP (n = 20)	–0.034	15.24	0.661
X_4 – C (n = 54)	–0.033	–10.65	0.666
X_5 – DA (n = 8)	0.016	12.28	0.831

Multiple correlation coefficient (R) = 0.263
* For abbreviations, see Table 1
n_1 and n For explanation, see Table 8

trols ($p > 0.05$) Correction for differences in age and body length did not substantially alter the relationship of pituitary weight to prostatic histology (Table 7)

In the course of the multiple regression analysis, simple correlation analysis was also performed, the correlation coefficients are presented in Table 8 No significant relationship between pituitary weight and variables referring to prostatic histology (X_2 to X_5) could be demonstrated

Multiple Regression Analysis

Whenever the stepwise procedure was performed with the total set of explanatory variables (X_2 to X_{15}), body length (X_{15}) was the only factor which significantly reduced the variance of pituitary weight ($p < 0.001$) The explanatory value of this factor, expressed as the square of the correlation coefficient (R^2) was 0.063 This means that 6.3 per cent of the variation in pituitary weight could be attributed to variations in body length

The full regression analysis was run with body length (X_{15}) and the variables for prostatic histology (X_2 to X_5) The results appear from Table 9 Among the histological diagnoses, none of the regression coefficients came near to statistical significance The regression coefficients for all groups of prostatic histology did not differ significantly from each other ($F = 0.3402$ $df_1 = 4$ $df_2 = 164$ $p > 0.25$) The regression coefficient for body length, on the other hand, was still highly significant ($p < 0.001$) The total set of variables included in the full regression analysis had an explanatory value of about 7 per cent ($R^2 = 0.069$), the histological diagnoses adding very little to the degree of explanation

COMMENT

If pituitary weight variation observed in the present study were approached by multiple regression analysis it was assumed that it might be possible to account for the most powerful confounding factor(s) thus allowing a more valid comparison of pituitary weight in groups presenting different prostatic histology

The present series provides no support for the prevailing opinion of pituitary involution in the ageing male (*Rasmussen* 1928 *Floderus* 1944) If tested together with other factors such as body length, age alone appeared to be of no significance in pituitary weight variability

Among the factors tested body length alone showed a significant relationship to pituitary weight variation This coincides with a similar observation by *Rasmussen* (1928) who showed that the *pars distalis* was responsible for the increased pituitary weight in tall people Hence in studies of pituitary weight

in man, corrections for body length differences may be more appropriate and important than corrections for age and body weight

Organ weights obtained at autopsy may be influenced and biased by many factors for which reason autopsy data should be analysed with care (*Calloway et al* 1965) As defined in the present study, no evidence was found to suggest that cause of death, duration of final illness, treatment with steroid hormones or the presence of liver cirrhosis had any major influence upon pituitary weight

The few diabetics in the present series appeared to have higher pituitary weights than non diabetics This observation may have occurred by chance as the number of patients examined was small However, in view of the increased frequency of BNH in patients with diabetes mellitus (*Bourke* & *Griffin* 1966), some form of pituitary hyperactivity in diabetics also leading to abnormal growth of the prostate cannot be excluded The diabetogenic action of growth hormone is now generally accepted (*Luft* 1966), and diabetes mellitus has been suggested as one factor in the pathogenesis of benign prostatic hyperplasia (*Roberts* 1967)

Previous studies (*Mellgren* 1945, *Moore* 1947, *Hartl* 1949, *Russfield* & *Byrnes* 1958) have failed to demonstrate abnormal pituitary weight in patients with advanced stages of BNH of the prostate, the reasons may be several In these studies pituitaries from patients with prostates of „normal size" were used as controls and some of the data were obviously collected from autopsy file records It has previously been shown that prostates showing „normal size and weight" often harbour foci of carcinoma and hyperplasia (*Harbitz* & *Haugen* 1972) and thus, normality should be based on thorough histological examination Similarily, haphazardly collected sections from autopsy files from so-called normal prostates should not be accepted since only a minor portion of the gland is likely to be represented *Russfield* & *Byrnes* (1958) found no differences in the pituitary weight among men with normal prostate, BNH or clinically manifest prostatic carcinoma Apart from dubious controls, several of their patients were either operated upon, castrated or treated with oestrogenic hormones, factors which all could have a considerable impact upon pituitary morphology or weight Thus, the negative conclusions made by others can therefore be seriously questioned

On the basis of the present data it was shown that the pituitary weight was maintained to a larger extent if BNH or carcinoma of the prostate could be demonstrated There was a significant decrease of pituitary weight with age in men exhibiting a histologically normal prostate In the 7th decade of life, men with benign hyperplasia and neoplasia of the prostate had significantly higher pituitary weights than age matched controls The present observations may therefore indicate that the pituitary gland can be involved, either primarily or secondarily, in the development of prostatic hyperplasia and neoplasia.

It emerged clearly from the multiple regression analysis, however, that pituitary weight as such cannot be used to discriminate between various forms of prostatic histology The low explanatory value of the variables tested may indicate failing linearity, but the importance of other unknown factors not included in the analysis remains open

In papers to follow, the cytology and the frequency of adenomas of the pituitary gland in relation to the histological appearence of the prostate and to other factors discussed here, will be reported

---

I am indebted to Professors *Helge Stalsberg*, M D and *Knut Westlund*, M D, Institute of Medical Biology, University of Tromso, Norway for helpful advice in matters concerning the statistical analysis and for reading the manuscript Actuary *Ingar Holme*, Cand real, The Norwegian Computing Center, Oslo, Norway, performed the computer analysis and gave valuable advice

fr
an
mers Bekjempelse

REFERENCES

1 *Armitage, P* Statistical methods in medical research Blackwell Scientific Publications, Oxford and Edinburgh 1971 p 384–391

2 *Asano, M* Basic experimental studies of the pituitary prolactin prostate inter relationships J Urol *93* 87–93, 1965

3 *Asano, M, Kanzaki, S, Sekiguchi, E & Tasaka, T* Inhibition of prostatic growth in rabbits with antiovine prolactin serum J Urol *106* 248–252, 1971

4 *Bourke, J B & Griffin, J P* Hypertension, diabetes mellitus, and blood groups in benign prostatic hypertrophy Brit J Urol *38* 18 23, 1966

5 *Calloway, N O, Foley, C F & Lagerbloom, P* Uncertainties in geriatric data. II Organ size J Amer Ger Soc *13* 20–28, 1965

6 *Floderus, S* Untersuchungen uber den Bau der menschlichen Hypophyse mit besonderer Berucksichtigung der quantitativen mikromorphologischen Verhaltnisse Acta path microbiol scand Suppl *53* 1–276, 1944

7 *Grayhack, J T* Pituitary factors influencing growth of the prostate In Vollmer, F P (Ed) Biology of the Prostate and Related Tissues Nat Cancer Inst Monogr *12* 189–199, 1963

8 *Harbitz, T B & Haugen, O A* Histology of the prostate in elderly men A study in an autopsy series Acta path microbiol scand Section A *80* 756–768, 1972

9 *Hartl, F* Anatomische Befunde an inkretorischen Drusen, insbesondere an der Hoden bei der sogenannte Prostatahypertrophie Zeitschr f Urol *42* 316–334, 1949

10 *Haugen, O A & Harbitz, T B* Prostatic weight in elderly men An analysis in an autopsy series. Acta path microbiol scand Section A *80* 769–777 1972

11 *Huggins, C & Russell P S* Quantitative effects of hypophysectomy on testis and prostate of dogs. Endocrinology *39* 1 7 1946

12 *Lostroh, A J & Li, C H* Stimulation of the sex accessories of hypophysectomized male rats by nongonadotrophic hormones of the pituitary gland Acta endocr (Kbh) *25* 1–16, 1957

13 *Lower, W E* Endocrine influence in the production of prostatic hypertrophy and a method for its control Amer J Surg *20* (II) 230–253, 1933

14 *Luft, R* The so called growth hormone of the anterior pituitary Acta med scand Suppl *445* 410–415, 1966

15 *Mellgren, J* The anterior pituitary in hyperfunction of the adrenal cortex An anatomical study with special reference to syndroma Morgagni and notes on prostatic hypertrophy Acta path microbiol scand Suppl *60* 1–177, 1945

16 *Moore, R A* Benign hypertrophy and carcinoma of the prostate In Twombly, G H & Pack G T (Eds) Endocrinology of Neoplastic Diseases A symposium by eighteen authors Oxford University Press, New York 1947 p 194–212

17 *Rasmussen, A T* The weight of the principal components of the normal male adult human hypophysis cerebri Am J Anat *42* 1–27, 1928

18 *Roberts H J* Pathogenesis of prostatic hyperplasia and neoplasia Geriatrics *22* 85–92 1967

19 *Russfield, B Agnes & Byrnes, R L* Some effects of hormone therapy and castration on the hypophysis in men with carcinoma of the prostate Cancer *11* 817–828 1958

20 *Scheffé, H* The analysis of variance John Wiley & Sons, Inc, New York 1959 p 25–51

21 *Snedecor, G W & Cochran, W G* Statistical methods 6th ed The Iowa State University Press Ames Iowa 1967 p 114–116

22 *Sommers S C* Endocrine changes with prostatic carcinoma Cancer *10* 345–358 1957

23 *Van der Laan W P* Observations on the hormonal control of the prostate gland Lab Invest *9* 185–190 1960

Acta path microbiol. scand Section A 81, 213-221, 1973

LIVER MORPHOLOGY AND GALLSTONE FORMATION IN HAMSTERS AND MICE TREATED WITH CHENODEOXYCHOLIC ACID

F BERGMAN and W VAN DER LINDEN

The Department of Pathology, University of Umeå, and the Surgical Department, Centrallasarettet, Östersund, Sweden

Hamsters fed a lithogenic diet showed a significantly stronger tendency to gallstone formation if the diet was supplemented with 0 5 per cent chenodeoxycholic acid In hamsters and in mice the addition of chenodeoxycholic acid to the diet resulted in a rapid and drastic shift towards this bile acid in the animals' bile Mice were fed normal chow with supplements of 1 per cent cholesterol, 1 per cent cholesterol plus *0 5 per cent cholic acid* and 1 per cent cholesterol plus 0 5 per cent chenodeoxycholic acid respectively After four months, slight fatty degeneration with occasional cholesterol crystals were observed in the livers of the animals of the first group In the second group there was marked fatty degeneration with a massive accumulation of cholesterol crystals In the third group, the liver histology was essentially normal Gallstones were present in the second group and absent in the other two In a regression experiment, hamsters were first fed chow supplemented with cholesterol and cholic acid After one month they were divided into two groups which were fed chow supplemented with 0 5 per cent chenodeoxycholic acid and unsupplemented chow respectively *When the animals were sacrificed 40 days later the* massive accumulation of cholesterol crystals, induced by the cholesterol cholic acid containing diet was still present in all animals fed chow In the hamsters fed chow supplemented with chenodeoxycholic acid there were far less such crystals. The conclusion is that with chow as the basic diet a chenodeoxycholic acid supplement aids the hamster in freeing itself from excess cholesterol On the other hand, inflammatory changes of the liver were present in many of the chenodeoxyholic acid treated hamsters while they were missing in the controls. The same experiment was repeated with a gallstone provoking diet as the basic diet during the regression period With this basic diet chenodeoxycholic acid did not have any effect on the accumulation of cholesterol crystals in the liver It did however result in the development of inflammatory changes in the liver comparable to those observed in animals fed lithocholic acid The results of these experiments perhaps suggest that the basic diet may be an important factor also in the clinical trials with chenodeoxycholic acid which are now carried out They also stress the importance of careful control of liver function and morphology during these trials.

Received 6 xi 72 Accepted 6 xi 72

Requests for reprints should be addressed to Professor *Frank Bergman*, Department of Pathology, University of Umeå, S 901 87 Umeå, Sweden

Studies of the animal model have contributed greatly to our present knowledge of gallstone formation Although the results obtained in different species and with different diets were

often conflicting, these studies have stressed the importance of dietary factors and emphasized the role of abnormal hepatic bile rather than that of a faulty gallbladder. Recent observations of a decreased bile acid pool size in gallstone patients together with speculations about an effect of chenodeoxycholic acid on this pool size have focused attention on this latter bile acid. In the present study we investigated the effect of chenodeoxycholic acid on gallstone formation in hamsters and mice, the animals most often used in studies of this kind. As prolonged feeding of cholic acid – the other primary bile acid – is known to lead to morphological changes in different organs, i.a. the liver, we also studied the effect of chenodeoxycholic acid on liver histology. This seemed the more important as therapeutic trials with this drug have now been started and there is some concern about its possible hepatic toxicity.

MATERIAL AND METHODS

Experiment I a

Sixteen (16) hamsters of both sexes were randomly divided into two equal groups. One group was given the gallstone provoking diet 284, for the composition of which the reader is referred to an earlier publication (*Bergman & van der Linden* 1967). The other group was given the same diet but supplemented with 0.5 per cent chenodeoxycholic acid. After 1 week on these two respective diets, the animals were operated in aether anaesthesia. The common bile duct was ligated in its terminal portion and enough time (about half an hour) allowed to elapse for the complete filling of the gallbladder. The gallbladder was then removed and incised and its contents were collected. In this way 4 samples of bile were collected in each group with 2 animals contributing to each sample. 0.05 ml of bile was extracted according to the method of *Wollenweber et al.* (1966). According to the same method the free bile acids were separated by thinlayer chromatography together with cholic acid, chenodeoxycholic acid and deoxycholic acid standards with the only modification that DC Fertig platten Kieselgel F_{54}* were used. After spraying with 15 per cent phosphomolybdic acid in aethanol the plates were heated to 110° C for 10 minutes. The spots were then quantified by means of a Vitatron Densitometer U.F.D.§ equipped with a 578 mμ filter and compared with standard curves. The Rf values were: cholic acid 0.17, chenodeoxycholic acid 0.42, and deoxycholic acid 0.47. For a discussion of the precision and accuracy of this method the reader is referred to an earlier publication (*Juul & van der Linden* 1969).

Experiment I b

Twenty four (24) hamsters of both sexes were randomly divided into two equal groups which were fed with the two respective diets described in Exp. I a. The animals were individually caged and had free access to food and water. After one month there were 9 survivors in the group fed the chenodeoxycholic acid supplemented diet and 11 surviving controls. These survivors were then sacrificed and examined as previously described in detail (*Bergman & van der Linden* 1967).

Experiment II a

Thirty (30) male mice with a mean weight of 16 g were randomly divided into three equal groups. The animals were individually caged and had free access to food and water. The three groups were fed vitaminized commercial chow† with the following dietary supplements: 0.5 per cent cholic acid plus 1.0 per cent cholesterol, 0.5 per cent

cholestero

After 1 [illegible]

animals were operated in the same way as the hamsters in Experiment I a. In each group 5 samples of bile were collected. These samples were investigated in the same way as those of Exp. I a.

Experiment II b

Forty-two (42) mice with a mean weight of 12 g were randomly divided into three equal groups which were fed the same diets as those of experiment II a. After 4 months on these three respective diets the mice were sacrificed. At autopsy the gallbladder was removed and opened permitting its contents to run out on blotting paper so that even small concretions could not escape detection. As in the earlier experiments the liver, gallbladder, lungs, heart, spleen, kidneys, adrenals and thyroid [illegible]

Frozen sections of liver, heart, kidneys and adrenals were stained with Scharlach Rot and Sudan Black B and also analysed with the polarizing microscope. The histological examinations were performed with the assessor unaware of the animals' group belonging.

* Merck, Darmstadt, Germany.

§ Vitatron, Dieren, Holland.

† Harald Fors, Holmsund, Sweden.

Experiment III

Twenty (20) hamsters of both sexes were fed

groups One group the controls were given un
fed
eno-
res
pective d ets the animals were sacrificed and investigated in the same way as the mice of the previous Experiment

Experiment IV

Twenty four (24) hamsters of both sexes were fed with commercial chow supplemented with 1 per cent cholesterol and 0 5 per cent cholic acid just as the hamsters of Experiment III After 1 month on this diet they were randomly divided into two equal groups One group was treated with the gallstone provoking diet 284 and the other group was given the same diet but supplemented with 0 5 per cent chenodeoxychol c acid After 40 days on these two respective diets the animals were sacrificed and investigated in the same way as those of Exp III

RESULTS

The findings in Exp I a appear from Fig 1 In the bile of the animals fed the gallstone inducing diet the mean cholic acid concentration was slightly higher than that of chenodeoxycholic acid In the hamsters fed a chenodeoxycholic acid supplement the opposite was the case chenodeoxycholic acid dominating the bile acid pattern

The results of Exp I b are shown in Table 1 As seen in this table cholesterol concretions were found in 2 out of the 11 survivors in the control group In the group fed the diet supplemented with 0 5 per cent chenodeoxycholic acid such concretions were present in 8 out of 9 animals This difference is statistically significant, $P = 0\ 005$ according to *Mainland et al* (1956) Liver histology revealed slight accumulation of cholesterol crystals in one animal in each group while signs of fatty degeneration were present in two animals of the experimental group and in one of the controls

The results of experiment II a appear from Fig 2 As seen in this Fig feeding a supplement of 1 per cent cholesterol resulted in a bile acid pattern which did not appreciably differ from that which is normally found in mice Cholic acid was the predominant bile acid in this group This predominance of cholic acid was even more accentuated in the group fed a supplement of cholesterol and cholic acid The difference between these two groups in the relative concentrations of the two primary bile acids was statistically significant with the Mann Whitney U test (*Siegel* 1956) In the group given a supplement of cholesterol and chenodeoxycholic acid this latter bile acid dominated strongly The difference from the group given only cholesterol was again significant if subjected to the same test

The results of the long term experiment II b are summarized in Table 2 As seen in this table feeding cholesterol and cholic acid resulted in fatty degeneration and massive accumulation of esterified cholesterol as birefractive crystals in the liver In the group fed cholesterol only fatty degeneration was far less pronounced and cholesterol crystals were only occasionally observed In the group fed

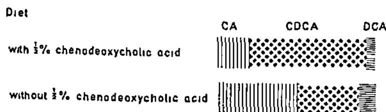


Fig 1 Biliary bile acid pattern in hamsters fed a gallstone provoking diet with and without 0 5 per cent chenodeoxychol c acid CA – cholic acid CDCA chenodeoxycholic acid DCA – deoxycholic aid

TABLE 1 *Concretions in Hamsters fed a Gallstone provoking Diet with and without Chenodeoxycholic Acid*

Diet	No	Cholesterol stones	Mixed stones	Pigmented stones	No stones
with chenodeoxycholic acid	9	8	0	0	1
without chenodeoxycholic acid	11	2	0	1	8

cholesterol and chenodeoxycholic acid liver histology was normal In the group fed a supplement of cholesterol and cholic acid, cholesterol gallstones were observed in all animals and the gallbladders in this group were as a rule distended In some animals the gallbladders showed small mucosal erosions and scattered inflammatory cells In the two other groups no gallstones were seen and the gall bladders had a normal appearance The thyroid gland of some of the mice fed the cholesterol cholic acid diet had a resting appearance and the adrenals revealed an increase of stainable lipids and birefractive cholesterol crystals in all layers of the cortex For a more detailed description of these morphological changes in the endocrinal glands the reader is referred to a previous study on gallstone

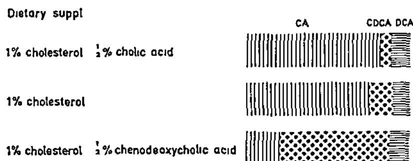


Fig 2 Biliary bile acid composition in mice fed chow with different supplement CA – cholic acid CDCA – chenodeoxycholic acid DCA – deoxycholic acid

TABLE 2 *Results of Different Diets in Mice*

Dietary supplement	Liver histology	Gallbladder	Gallstones
1 per cent cholesterol 0 5 per cent cholic ac	Fatty degeneration massive accumulation of cholesterol crystals	Distended	Present
1 per cent cholesterol	Slight fatty degeneration occasional cholesterol crystals	Normal	Absent
1 per cent cholesterol 0 5 per cent chenodeoxycholic acid	Normal	Normal	Absent

TABLE 3 *Liver Histology of Hamsters which after Treatment with Cholesterol and Cholic Acid were fed Chow with and without Chenodeoxycholic Acid Supplement*

	Cholesterol crystals				Fatty degeneration				Inflammation		Σ
	+ + +	+ +	+	−*	+ + +	+ +	+	−*	+	−§	
without suppl	10	0	0	0	5	5	0	0	0	10	10
with suppl	0	6	2	2	4	4	1	1	6	4	10

* + + + massive + + moderate + occasional, − absent
§ + present − absent

formation in hamsters and mice (Bergman *et al* 1970) Apart from a slight increase of lipids in the adrenal cortex in mice fed cholesterol only, the endocrinal glands had a normal appearance in the two other groups The histological examination of the other organs revealed no systematic changes in any group

In the first regression experiment (Exp III) the hamsters in the control group – which after treatment with the cholesterol-cholic acid supplemented diet had been given normal chow – all had a conspicuous enlargement of the liver In these animals the liver had the pale yellow colour typical of extreme steatosis At microscopy there was a massive accumulation of intra cellular lipid droplets and birefractive cholesterol crystals in the parenchymal and Kupffer's cells In the ani-

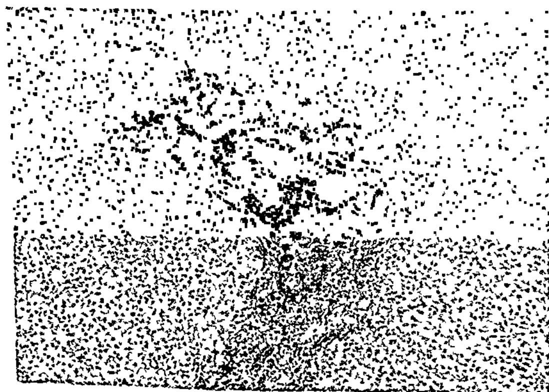


Fig 3 Liver of hamster fed a gallstone provoking diet with 0.5 per cent chenodeoxycholic acid 40 days after 1 month's pretreatment with cholesterol-cholic acid Widening of a portal space with hyperplasia and dilatation of small bile ducts. Marked accumulation of inflammatory cells Van Gieson's stain × 80

TABLE 4 *Liver Histology of Hamsters which after Treatment with Cholesterol and Cholic Acid were fed a Lithogenic Diet with and without Chenodeoxycholic Acid Supplement*

	Cholesterol crystals				*Fatty degeneration*				*Inflammation*		Σ
	+ + +	+ +	+	–*	+ + +	+ +	+	–*	+	–§	
without suppl	8	3	0	1	10	1	0	1	0	12	12
with suppl	5	5	2	0	8	4	0	0	12	0	12

* + + + massive, + + moderate, + occasional, – absent
§ + present, absent

mals treated with chenodeoxycholic acid, on the other hand, the livers were macroscopically far more normal The histological picture was dominated by the accumulation of intracellular fat, but cholesterol crystals were absent in 2 animals and in none of the others the aggregates of such crystals were as massive as in the controls (See Table 3) On the other hand in 6 of the hamsters treated with chenodeoxycholic acid, but in none of the controls, there were severe pathological changes of the liver of the same type as those described by *Drews* (1963) ($P = 0.01$ according to *Mainland et al* 1956) These changes – which Drews originally thought to be related to gallstone formation in the hamster – resemble the "Begleitcholangitis" (*Franke 1955*) of human pathology The portal spaces were noticeably widened with proliferation of the small bile ducts and accumulation of inflammatory cells, often eosinofilic granulocytes No "bile thrombi" were observed, but some ducts contained amorphous basophilic material associated with damaged ductal cells and eosinofilic granulocytes (Fig 3)

Finally in regression experiment IV in which we used a gallstone provoking diet as the basic diet all the hamsters of the control group with one exception and all those fed a diet supplemented with chenodeoxycholic acid, still had all the signs of an extreme steatosis of the liver As shown in Table 4 there was no difference between the two groups as to the presence of cholesterol crystals in the liver Liver histology revealed, however, one highly significant group difference ($P < 0.001$) Pathological changes of the liver of the same type as those found in the chenodeoxycholic acid treated hamsters of Exp III were found in all the hamsters fed this acid, but in none of the controls Finally the gallbladders were extremely distended in the animals treated with chenodeoxycholic acid and gallstones were absent in both groups

DISCUSSION

In a comparative study of the bile acid pattern of hamsters fed different diets (*Bergman et al* 1968) we found a relatively high concentration of chenodeoxycholic acid in the bile of hamsters fed gallstone provoking diets On the other hand, there was more cholic acid in the bile of animals on antilithogenic diets In pilot Exp I a we had found that the administration of chenodeoxycholic acid resulted in a rapid and drastic shift towards this acid in the hamster's bile This was our original reason for studying the effect of chenodeoxycholic acid supplement on the animal's tendency to gallstone formation The results of experiment I b show that such a supplement intensifies this tendency markedly (See Table 1) Studies of gallstone formation in hamsters are often complicated by the variability of the hamsters reaction to the diet As the results of the control group show, the hamsters used in Exp I b were much less prone to form gallstones on this diet than otherwise seen to be the case In the group given the chenodeoxycholic acid supplement however all but one of the animals formed cholesterol concretions These results are in accordance with independent studies

by *Dam et al* (in press) who found that the addition of only 0 1 per cent chenodeoxycholic acid produced cholesterol gallstones in hamsters reared on a gallstone preventing diet The results are also in agreement with our earlier observation in the hamster gall stone model that gallstone formation often seems to be accompanied by a shift towards chenodeoxycholic acid

In Tepperman s mouse model (*Tepperman et al* 1964), however, we met with totally different results In Exp II a we again found that dietary supplements of primary bile acids result in fast and drastic shifts in the bile acid pattern Normally cholic acid is the predominant bile acid in mice (*Danielsson & Kazumo* 1959) Whenever cholic acid was added to the diet this dominance was even more accentuated With chenodeoxycholic acid, on the other hand the opposite was observed These fast shifts in bile acid composition which we observed in both species are probably due to a strongly diminished neosynthesis of bile acids as the result of the continuous supply of these acids to the liver from the intestine (*Bergström & Danielsson* 1958) Feeding cholesterol during one week did not result in any significant change in the ratio between the primary bile acids These results may at first sight seem to be at variance with those reported by *Beher et al* (1970) in rats In these animals a cholesterol supplement to the diet resulted in a decrease of the cholic acid and an increase of the chenodeoxycholic acid concentration It is possible that the reason for this discrepancy is a difference in the experimental period Our experiments lasted only for one week as compared with three weeks in the study by *Beher et al* (1970)

Contrary to findings in the hamster model chenodeoxycholic acid did not provoke gall stones in the mouse Moreover it even protected the liver against the accumulation of cholesterol crystals and fatty degeneration Whenever the diet was supplemented with cholesterol and cholic acid a massive accumulation of such crystals would be found while cholesterol gallstones were observed in the distended gallbladders With a cholesterol – chenodeoxycholic acid supplement, on the other hand, gallstones were absent and liver histology was normal Not even signs of fatty degeneration were present although such changes appeared in a number of animals fed 1 per cent cholesterol only

In earlier studies (*Bergman et al* 1970) hamsters were fed chow supplemented with cholesterol and cholic acid and we found similar changes in liver histology i e fatty degeneration and accumulation of cholesterol crystals, as in the mouse In the mouse, discontinuance of the diet resulted in a rapid return to normal of liver histology The hamster, however, appeared to be unable to free itself from the accumulation of cholesterol in the liver Even after 2 months on normal chow the histological picture remained largely unchanged The results of the regression experiment (Exp III) show that the addition of chenodeoxycholic acid to normal chow greatly helps the hamster to free itself from excess liver cholesterol In 2 animals fed this supplement cholesterol crystals were completely absent, in the rest such crystals were by far less numerous than in the controls fed unsupplemented chow The results of Exp II b and III show that with chow as the basic diet chenodeoxycholic acid protects the animal against cholesterol accumulation and enhances its excretion *Beher et al* (1970) found two factors to be responsible for an increased elimination of accumulated tissue cholesterol 1 An increased bile acid turnover rate resulting in a higher rate of conversion of cholesterol to bile acids 2 A shift in the bile acid spectrum towards chenodeoxycholic acid the acid which they found to have a higher turnover rate A similar shift towards chenodeoxycholic acid which we then were unable to explain did we meet in an earlier study of the regression of cholesterol accumulation in hamster (*Bergman et al* 1970) Clearly if this it correct the addition of chenodeoxycholic acid to the diet with its subsequent drastic shift towards this acid in the bile acid pool pattern will be very helpful in eliminating excess cholesterol But as

lymforeticular proliferations The *demonstration of specific granulomas* or *tumour metastasis* in histo logical specimens might also lead to a final diagnosis in patients presenting an obscure clinical picture.

I Dahl, L Angervall & J. Save-Söderbergh
ATYPICAL FIBROBLASTIC TUMOURS (FIBROSARCOMA) IN EARLY INFANCY

Fibrosarcoma occurring in early infancy (children less than 3 years of age) is extremely rare The presented series comprises 8 children (7 girls and 1 boy) The tumour was observed at birth in 3 children All patients were treated surgically—one received radiation pre-operatively and another post-operatively Re-operation for early recurrence was performed on 2 patients All the patients were free from further recurrence and/or signs of me tastases after 1–25 years of observation

In 5 cases the infiltratively growing tumour was very cellular and rich in mitoses The extracellular amount of collagen varied but was generally sparse Electron microscopical studies in 2 of these cases supported our opinion that the tumours were composed of immature fibroblasts In the remaining 3 cases the tumours were rich in collagen, the cells were more polymorphous and showed a fascicular arrangement i e an appearance more like that of fibrosarcoma in adults

We consider it possible to divide fibrosarcomas in early infancy into 2 forms, medullary and desmoplastic fibrosarcoma. With respect to the benign clinical course in our series as well as in single *tentative cases reported in the literature, atypical* fibroblastic tumour in early infancy is perhaps a better term than medullary fibrosarcoma. We prefer the term fibrosarcoma, however, as long as larger series have not yet definitely proven the benignity of the tumour

L G Kindblom, L Angervall & L Enerbäck
HISTOCHEMICAL DIFFERENTIATION OF MUCOSUBSTANCES IN MESENCHYMAL TUMOURS

Mucosubstances constitute an important fraction of the ground substance and matrix of the intercellular spaces in connective tissue Some of these mucosubstances have been isolated and characterized biochemically Differentiation of acid mu copolysaccharides in connective tissue has been accomplished by Scott et al utilizing the critical electrolyte concentration phenomenon Alcian blue stains the known mucopolysaccharides up to a cri

[illegible]

in myxoid and chondroid tumours and chordomas

Tissue controls with biochemically characterized mucopolysaccharides were used ganglion (hyaluronic acid), foetal cartilage (chondroitin 4 and -6 sulphate), mast cells (heparin), adult cartilage (keratan sulphate) and nucleus pulposus with notochordal tissue (keratan sulphate)

Fifty-five tumours were examined The mu cosubstance in myxoma and myxoid liposarcoma stained up to the same critical electrolyte concentration as the control tissue containing hyaluronic acid, chondromatosis in bursa, tendon and synovia and well differentiated chondrosarcoma showed the same staining pattern as keratan sul phate in adult cartilage, low differentiated skeletal chondrosarcoma and extraskeletal and mesenchymal chondrosarcoma stained as foetal cartilage containing chondroitin sulphates The mucosubstance in chordoma stained as notochordal tissue (keratan sulphate) in nucleus pulposus, which gives support for the opinion that chordoma takes origin from notochordal tissue

G Hansson & J Save Söderbergh
"CORPORA AMYLACEA" IN THE PARATHYROIDS IN PRIMARY HYPERPARATHYROIDISM

Intrafollicular amyloid deposition in parathyroids from cases operated on for primary hyperpara thyroidism has recently been described We therefore studied surgically removed parathyroids from 108 cases of primary hyperparathyroidism, com prising 93 cases with adenomas, 11 with water clear cell hyperplasia and 4 with chief cell hyper plasia of the glands. In 87 out of the 108 cases the enlarged glands contained colloidfilled follicular structures, mostly built up by dark and light chief cells Oxyphil and transitional cell forms were also seen In 17 cases, all of which were adenomas, intrafollicular deposits stained by alkaline Congo red were found The deposits showed a green birefringence, sometimes in the form of a Maltese Cross If viewed in the electron microscope, such intrafollicular material from one case was partly made up by parallel oriented fibres and contained

[illegible]

carcinoma

Our study shows that the follicular structure is a prerequisite for a formation of amyloid staining deposits, possibly formed by altered colloid and cell detritus The histochemical and polarization optical similarity to corpora amylacea in the prostate and lungs seems to make this term a proper designation for the intrafollicular deposits Their fibrillar character probably explains the amyloid like birefringent character in Congo red stain The further relationship between this material and amyloid in e g medullary carcinoma is not clear and requires further study

Acta path microbiol scand Section A 81, 3, 225–228, 1973

THE ADRENAL GLANDS OF MICE WITH HEREDITARY PITUITARY DWARFISM

JOHN G M SHIRE and ELIZABETH A HAMBLY

Institute of Genetics, University of Glasgow, Glasgow, Scotland
and Department of Genetics, University of Cambridge, England

Histological studies were made of the adrenal glands of mice with hereditary pituitary dwarfism. No evidence was found for a deficiency of ACTH in these mice Quantitative comparisons with their normal sibs showed that the permanent cortex was not disproportionately small in the dwarf mice It was confirmed that the adrenal glands of dwarf mice lack an X zone The medulla was found to be large, relative to the permanent cortex, in both male and female dwarfs.

The dwarf mouse was described by *Snell* (1929) who showed that the syndrome was caused by the Mendelian recessive, *dw* The pituitary gland is the primary site of action of the mutant gene (*Carsner & Rennels* 1960) There is direct evidence that the mutant pituitaries are deficient in growth hormone (*Lewis* 1967) and in prolactin (*Bartke* 1967, *Cheever et al* 1969) The production of thyrotropin is reduced (*Ortman* 1956, *Wegelius* 1959) but the gonadotropins are unaffected (*Smith & MacDowell* 1931) A deficiency of corticotropin (ACTH) has been suggested (*Hadorn* 1961, *Lewis* 1967) because of descriptions of the adrenals as underdeveloped (*Smith & MacDowell* 1930, *Kemp & Marx* 1937 *Bartels* 1941, *Gruneberg* 1952) In this report we present quantitative data on the structure of the adrenals of dwarf mice and discuss the earlier observations on them

Received 14 xii 72 Accepted 14 xii.72

Requests for reprints should be addressed to *J G M Shire* Institute of Genetics, University of Glasgow Glasgow G11 5JS, Scotland.

Lecture given at the University of Oslo on Dec 14 1972

MATERIALS AND METHODS

The stock of mice segregating for *dw* was obtained

mates, aged eight weeks, were weighed and then killed with ether Their adrenals were fixed in Bouin's fluid Some were cleaned of adherent fat and weighed on a torsion balance before fixation Serial sections 6 μm thick, stained with haematoxylin and eosin, were made from each adrenal The volumes of the medulla, permanent cortex (zona fasciculata plus zona glomerulosa) and X zone (if present), were measured for each adrenal by the method described earlier (*Shire & Spickett* 1968) The values for the left and right adrenals were added together to give the total volume of each tissue for each mouse.

RESULTS

Histological examination showed that the adrenals of dwarf mice were easily damaged by cleaning them before weighing, but that those of normal mice were not When corrected for differences in body weight, the weights of undamaged adrenals from both male and female dwarfs were similar to those from nor-

TABLE 1 *Mean (± S E) Absolute and Relative Weights of Undamaged Adrenals from Dwarf Mice and Their Normal Sibs*

	Males	Females
Adrenal wt (mg)		
Normal	3 9 ± 0 2	9 1 ± 0 5
Dwarf	1 4 ± 0 2	1 2 ± 0 1
mg adrenal/100 g of body		
Normal	13 0 ± 0 6	32 9 ± 1 0
Dwarf	15 6 ± 1 7	13 3 ± 0 3

TABLE 2 *The Mean (± S E) Volume of the Permanent Cortex (PC) and Body Weight in Dwarf Mice and Their Normal Sibs*

	Body wt g	PC volume mm^3	mm^3 PC/100 g of body
Normal			
Males	30 2 ± 0 7	1 43 ± 0 10	4 7 ± 0 3
Females	27 6 ± 1 0	2 57 ± 0 25	9 3 ± 0 9
Dwarf			
Males	9 3 ± 0 7	0 57 ± 0 06	6 0 ± 0 3
Females	9 0 ± 0 5	0 57 ± 0 06	6 4 ± 0 5

mal males (Table 1) The adrenals of the normal females were heavier than those of any of the other mice, partly because they contained large, cortical, X zones undergoing fatty degeneration (*Shire & Spickett* 1968) The X zone was absent from the adrenals of the post pubertal normal males, and was only a few cells wide in both male and female dwarfs, as had been described earlier (*Dean esly* 1938, *Bartels* 1941) No differences in the zona glomerulosa were noted between dwarf and normal mice, supporting previous sug gestions about electrolyte metabolism (*Lewis* 1967, *Stewart* 1967)

Quantitative measurements were made on the undamaged adrenal glands of eight male and eight female mice of the two phenotypes The relative volume of the permanent cortex in male and female dwarf mice was larger than that of the normal male mice, but smaller than that of the normal females (Table 2) A sex difference in cortical volume is present in most strains of adult mice (*Chester Jones* 1957, *Shire & Spickett* 1968) The relative volume of the medulla was the same for male and female dwarfs, and for their normal female sibs (Table 3) All three values were markedly greater than that for the normal males Sex differences in the volume of the medulla have been found in some but not *all*, strains of healthy mice (*Shire & Spickett* 1968)

DISCUSSION

The permanent cortex is the only part of the adrenal for which ACTH is a trophic hormone The quantitative data clearly show that the permanent cortex is not disproportionately small in dwarf mice The observed aplasia of the X zone in the dwarf mice is not a sign of ACTH deficiency for this hormone does not have a trophic action on this zone (*Chester Jones* 1957, *Shire & Spickett* 1968, *Shire & Stewart* 1972) Thus there is no evidence for a deficiency of ACTH in these mice contrary

TABLE 3 *The Mean (± S E) Volume of the Medulla (M) and Its Relation to Body Weight for the Mice in Table 2*

	M volume mm^3	mm^3 M/100 g of body	M volume as per cent of PC volume
Normal			
Males	0 31 ± 0 02	1 0 ± 0 1	22 ± 1
Females	0 54 ± 0 04	2 0 ± 0 2	21 ± 1
Dwarf			
Males	0 20 ± 0 02	2 2 ± 0 1	36 ± 2
Females	0 17 ± 0 02	1 9 ± 0 2	31 ± 3

to the impression given by several reviews of the dwarf phenotype (*Gruneberg* 1952, *Hadorn* 1961, *Lewis* 1967, *Dunn* 1970)

In male mice of most strains the volume of medulla bears a fairly constant relation to the volume of the permanent cortex (*Shire & Spickett* 1968, *Shire* 1970) However, the dwarf mice had medullae that were larger, in proportion to the permanent cortex, than those of their normal sibs (Table 3) In histological preparations of the adrenals of dwarf mice this makes the cortex appear narrow This could account for earlier, qualitative, descriptions of the cortex as 'thin' (*Smith & MacDowell 1930, Kemp & Marx 1937*), as would the effective absence of the X zone

The differences in the X zone, together with known sex differences in the volumes of the other parts of the adrenal, can account for our data on adrenal weight, and for those of *Smith & MacDowell* (1930) The other report (*Kemp & Marx* 1937) of reduced adrenal weight in dwarf mice was based on a very small sample The adrenals from those dwarfs may have been as susceptible to damage during cleaning as those of our dwarfs were.

REFERENCES

Bartels E D Studies on hereditary *dwarfism in mice* III Development of the adrenals in dwarf mice. Acta path microbiol. scand. *18* 20–35, 1941

Bartke, A Prolactin deficiency in genetically sterile dwarf mice Mem. Soc. Endocr *15* 193–197, 1967

Carsner, R L & Rennels, E G Primary site of gene action in anterior pituitary dwarf mice Science *131* 829, 1960

Cheever, E V, Seavey, B K & Lewis, U J Prolactin of normal and dwarf mice Endocrinology *85* 698–703, 1969

Chester Jones, I The adrenal cortex Cambridge University press, Cambridge 1957

Deanesly, R Adrenal cortex differences in male and female mice Nature Lond *141* 79, 1938

Dunn, T B Normal and pathologic anatomy of the adrenal gland of the mouse, including neoplasms J Nat Canc. Inst *44* 1323–1389, 1970

Gruneberg, H The genetics of the mouse, 2 ed Martinus Nijhoff, The Hague 1952

Hadorn E Developmental genetics and lethal factors Methuen Ltd, London 1961

Kemp T & Marx L Beeinflussung von erblichen, hypophysarem Zwergwuchs bei Mausen durch verschiedene Hypophysanauszuge und Thyroxin. II Endokrine Organe Acta path. microbiol scand *14* 197–227, 1937

Lewis, U J Growth hormone of normal and dwarf mice Mem Soc Endocr *15* 179–188, 1967

Ortman R A study of some cytochemical reactions and of the hormone content of the adenohypophysis in normal and genetic dwarf mice J Morph 99 417–431 1956

Shire J G M Genetic variation in adrenal structure quantitative measurements on the cortex and medulla of hybrid mice J Endocr *48* 419–431 1970

Shire, J G M & Spickett S G Genetic variation in adrenal structure strain differences in quantitative characters J Endocr *40* 215–229 1968

Shire, J G M & Stewart J The zona glomerulosa and corticotrophin a genetic study in mice J Endocr *55* 185–193, 1972

Smith, P E. & MacDowell E C An hereditary

15*

TABLE 1 *Mean (± S E) Absolute and Relative Weights of Undamaged Adrenals from Dwarf Mice and Their Normal Sibs*

	Males	Females
Adrenal wt (mg)		
Normal	3 9 ± 0 2	9 1 ± 0 5
Dwarf	1 4 ± 0 2	1 2 ± 0 1
mg adrenal/100 g of body		
Normal	13 0 ± 0 6	32 9 ± 1 0
Dwarf	15 6 ± 1 7	13 3 ± 0 3

TABLE 2 *The Mean (± S E) Volume of the Permanent Cortex (PC) and Body Weight in Dwarf Mice and Their Normal Sibs*

	Body wt g	PC volume mm^3	mm^3 PC/100 g of body
Normal			
Males	30 2 ± 0 7	1 43 ± 0 10	4 7 ± 0 3
Females	27 6 ± 1 0	2 57 ± 0 25	9 3 ± 0 9
Dwarf			
Males	9 3 ± 0 7	0 57 ± 0 06	6 0 ± 0 3
Females	9 0 ± 0 5	0 57 ± 0 06	6 4 ± 0 5

mal males (Table 1) The adrenals of the normal females were heavier than those of any of the other mice, partly because they contained large, cortical, X zones undergoing fatty degeneration (*Shire* & *Spickett* 1968) The X zone was absent from the adrenals of the post pubertal normal males and was only a few cells wide in both male and female dwarfs, as had been described earlier (*Dean esly* 1938, *Bartels* 1941) No differences in the zona glomerulosa were noted between dwarf and normal mice supporting previous sug gestions about electrolyte metabolism (*Lewis* 1967, *Stewart* 1967)

Quantitative measurements were made on the undamaged adrenal glands of eight male and eight female mice of the two phenotypes The relative volume of the permanent cortex in male and female dwarf mice was larger than that of the normal male mice, but smaller than that of the normal females (Table 2) A sex difference in cortical volume is present in most strains of adult mice (*Chester Jones* 1957 *Shire* & *Spickett* 1968) The relative volume of the medulla was the same for male and female dwarfs, and for their normal fe male sibs (Table 3) All three values were markedly greater than that for the normal males Sex differences in the volume of the medulla have been found in some but not all, strains of healthy mice (*Shire* & *Spickett* 1968)

DISCUSSION

The permanent cortex is the only part of the adrenal for which ACTH is a trophic hor mone The quantitative data clearly show that the permanent cortex is not disproportionately small in dwarf mice The observed aplasia of the X zone in the dwarf mice is not a sign of ACTH deficiency for this hormone *does not* have a trophic action on this zone (*Chester Jones* 1957, *Shire* & *Spickett* 1968 *Shire* & *Stewart* 1972) Thus there is no evidence for a deficiency of ACTH in these mice contrary

TABLE 3 *The Mean (± S E) Volume of the Medulla (M) and Its Relation to Body Weight for the Mice in Table 2*

	M volume mm³	mm³ M/100 g of body	M volume as per cent of PC volume
Normal			
Males	0 31 ± 0 02	1 0 ± 0 1	22 ± 1
Females	0 54 ± 0 04	2 0 ± 0 2	21 ± 1
Dwarf			
Males	0 20 ± 0 02	2 2 ± 0 1	36 ± 2
Females	0 17 ± 0 02	1 9 ± 0 2	31 ± 3

to the impression given by several reviews of the dwarf phenotype (*Gruneberg* 1952, *Hadorn* 1961, *Lewis* 1967, *Dunn* 1970)

In male mice of most strains the volume of medulla bears a fairly constant relation to the volume of the permanent cortex (*Shire* & *Spickett* 1968, *Shire* 1970) However, the dwarf mice had medullae that were larger, in proportion to the permanent cortex, than those of their normal sibs (Table 3) In histological preparations of the adrenals of dwarf mice this makes the cortex appear narrow This could account for earlier, qualitative, descriptions of the cortex as "thin" (*Smith* & *MacDowell* 1930, *Kemp* & *Marx* 1937), as would the effective absence of the X zone.

The differences in the X zone, together with known sex differences in the volumes of the other parts of the adrenal, can account for our data on adrenal weight, and for those of *Smith* & *MacDowell* (1930) The other report (*Kemp* & *Marx* 1937) of reduced adrenal weight in dwarf mice was based on a very small sample The adrenals from those dwarfs may have been as susceptible to damage during cleaning as those of our dwarfs were

REFERENCES

Bartels E D Studies on hereditary dwarfism in mice III Development of the adrenals in dwarf mice Acta path microbiol. scand *18* 20–35, 1941

Bartke, A Prolactin deficiency in genetically sterile dwarf mice. Mem. Soc Endocr *15* 193–197, 1967

Carsner, R L & *Rennels, E G* Primary site of gene action in anterior pituitary dwarf mice Science *131* 829, 1960

Cheever, E V, *Seavey, B K* & *Lewis, U J* Prolactin of normal and dwarf mice Endocrinology *85* 698–703, 1969.

Chester Jones, I The adrenal cortex. Cambridge University press, Cambridge 1957

Deanesly, R Adrenal cortex differences in male and female mice Nature Lond *141* 79, 1938

Dunn, T B Normal and pathologic anatomy of the adrenal gland of the mouse, including neoplasms J Nat Canc. Inst *44* 1323–1389, 1970

Gruneberg, H The genetics of the mouse, 2 ed. Martinus Nijhoff, The Hague 1952

Hadorn, E Developmental genetics and lethal factors Methuen Ltd, London 1961

Kemp, T & *Marx, L* Beeinflussung von erblichen, hypophysarem, Zwergwuchs bei Mausen durch verschiedene Hypophysanauszuge und Thyroxin. II Endokrine Organe Acta path. microbiol scand *14* 197–227, 1937

Lewis U J Growth hormone of normal and dwarf mice Mem Soc Endocr *15* 179 188 1967

Ortman, R A study of some cytochemical reactions and of the hormone content of the adenohypophysis in normal and genetic dwarf mice J Morph *99* 417–431, 1956

Shire, J G M Genetic variation in adrenal structure quantitative measurements on the cortex and medulla of hybrid mice J Endocr *48* 419–431, 1970

Shire, J G M & *Spickett, S G* Genetic variation in adrenal structure strain differences in quantitative characters J Endocr *40* 215–229, 1968

Shire, J G M & *Stewart, J* The zona glomerulosa and corticotrophin a genetic study in mice J Endocr *55* 185–193, 1972

Smith, P E. & *MacDowell, E C* An hereditary

anterior pituitary deficiency in the mouse Anat Rec *46* 249–257, 1930

Smith, P E & MacDowell, E C The differential effect of hereditary mouse dwarfism on the anterior pituitary hormones. Anat Rec *50* 85–93, 1931

Snell G D Dwarf, a new Mendelian recessive character of the house mouse Proc nat Acad Sci Wash *15* 733 734, 1929

Stewart, J In Discussion Mem. Soc Endocr *15* 188 189 1967

Wegelius, O The dwarf mouse–an animal with secondary myxedema. Proc Soc exp Biol Med *101* 225–227, 1959

Acta path. microbiol. scand. Section A. 81, 3, 229–232, 1973

THE EFFECT OF CYCLIC AMP ON THE ERYTHROPOIESIS IN MICE (NMRI)

A. JAKOBSEN and E. B. THORLING

Cancer Research Institute, Radiumstationen, DK 8000 Aarhus C. Denmark
Head: J. Bichel

The effect of N^6-2' O-dibutyryl cyclic AMP (db cyclic AMP) on erythropoiesis in NMRI mice was investigated. This effect was measured by the incorporation of ^{59}Fe into peripheral blood, spleen, and bone marrow. The study included plethoric as well as normal mice. Route of administration and dose schedule were varied. In spite of this, we were unable to demonstrate any stimulating effect of db-cyclic AMP on erythropoiesis.

In recent years cyclic AMP has been well established as a mediator of a variety of biological effects (*Robison et al.* 1971). It has been reported recently (*Schooley & Mahlmann* 1971) that N^6 2' O dibutyryl cyclic AMP (db cyclic-AMP) is able to stimulate erythropoiesis in the plethoric mouse. The present study was undertaken to determine whether this effect could be shown in other strains of mice and to investigate whether this effect could be explained by a stimulating effect on erythropoietin production in the kidneys.

MATERIALS AND METHODS

NMRI mice weighing about 20 g were used. They were made plethoric by hypoxia for 4 weeks at 0.4 atmosphere's pressure and used 6 days after removal from the hypoxic chamber. This method is thoroughly described elsewhere (*Thorling* 1970). The mice were injected with 6 μmol db-cyclic AMP (Boehringer M. Catalogue No. 15205) dissolved in isotonic glucose. Forty-eight hours after the first db-cyclic AMP injection the mice were injected subcutaneously with 0.5 μCi ^{59}Fe. Forty-eight hours later the mice were bled, and the percentage of ^{59}Fe incorporated in 1.0 ml of blood was determined. The percentage of the injected radioactivity incorporated into the spleen and femur was also determined. The following groups of mice were studied.

Group A

Six mice were injected intravenously with 4 x 0.2 ml of glucose containing 6 μmol db-cyclic AMP in 4 injections given at 8 hours' intervals. Six other mice injected intravenously with 4 x 0.2 ml of isotonic glucose served as controls.

Group B

Thirteen mice were injected intraperitoneally with a total of 0.8 ml of glucose containing 6 μmol db-cyclic AMP in 4 injections given at 8 hours' intervals. A further 13 mice were injected with a total of 0.8 ml of glucose containing 6 μmol db-cyclic AMP in 4 injections at 2 hours' intervals. The study includes furthermore a control group of 6 mice receiving 4 x 0.2 ml of isotonic NaCl.

Group C

Ten normal mice (5 ♂ and 5 ♀) were injected intraperitoneally with a total of 0.8 ml of glucose containing 6 μmol db-cyclic AMP in 4 injections at 2 hours' intervals. Ten other normal mice received 4 x 0.2 ml of glucose.

Group D

Eight mice were injected intraperitoneally with a total of 0.8 ml of glucose containing 6 μmol db-cyclic AMP in 4 injections at 8 hours' intervals. The same mice received a total of 0.2 ml of NaCl subcutaneously containing 2 mg of a freeze-dried

Received 7.viii.72. Accepted 14.xii.72.

TABLE 1 *Erythropoietic Response of Plethoric Mice to Injection of 6 μmol db cyclic AMP*

	No of animals	48 hr ^{59}Fe incorp in blood	48 hr ^{59}Fe incorp in femur	48 hr ^{59}Fe incorp in spleen
ıtrols ml glucose or NaCl or ı p	17	0 085 (0 069 0 119)*	0 122 (0 090 0 0151)	0 235 (0 207 0 358)
cyclic AMP mol ı v	6	0 151 (0 071 0 382)	0 170 (0 102 0 278)	0 252 (0 206 0 305)
cyclic AMP mol ı p 8 hrs intervals	13	0 165 (0 098 0 336)*	0 092 (0 073 0 135)	0 222 (0 184-0 235)
cyclic AMP mol ı p 2 hrs intervals	13	0 140 (0 061 0 207)	0 107 (0 091 0 135)	0 231 (0 168-0 258)
thropoietin standard	8	6 282 (3 987 9 486)	0 261 (0 079 0 418)*	1 103 (0 681 1 652)
thropoietin standard db-cyclic AMP mol ı p	8	6 914 (5 113 9 698)	0 397 (0 281 0 544)*	1 150 (0 788 1 564)

The values are given as the median for each group The numbers in parenthes s are the 95 per cent confidence limits The different groups were compared with the respective control groups using Mann Wh t ney s test

* Indicates that the underlying distributions are significantly different ($p < 0\ 05$) The other groups do not differ from the control groups at a 5 per cent level

* $p < 0\ 05$

urinary erythropoietin preparation (eqv to 0 75 international units) in 4 injections at 8 hours in tervals Eight mice receiving the same amount of erythropoietin served as controls The study includes furthermore a control group of 5 mice receiving 4 x 0 2 ml of isotonic NaCl

RESULTS

The results obtained in the present study are represented in Tables 1 and 2 Table 1 shows the percentage incorporation of radioactive iron into 1 ml of peripheral blood, femoral bone marrow and spleen of plethoric mice The results are given as the median value with the 95 per cent confidence limits in parenthesis The group were compared using Mann-Whitney s test (Documenta Geigy Wissenschaftliche Tabellen 1960) Two groups were found to differ significantly from the controls at a 5 per cent level Both values are in favour of db-cyclic AMP, but in one of the cases it can hardly be of any biological significance as the corresponding values in bone marrow and spleen are not different from the control values. In the other case in which a small difference was found in the iron incorporation into the femur the corresponding blood value was not different from the control value *The other groups do not differ significantly* from the controls ($p > 0\ 05$)

The lack of effect is apparently not dependent on the dose schedule used since 2 as well as 8 hours intervals gave the same results Also the route of administration seems of no importance since intraperitoneal and intravenous administration was equally *ineffective* Experiments were conducted in normal mice using the same procedures and again there was a complete lack of enhancing effect on the *erythropoiesis by the cyclic AMP* The values obtained for femur and spleen in male and female mice show consistent sex differences but there was no difference in results

TABLE 2 *Response of Normal Mice to Injection of 6 μmol db cyclic AMP*

	No of animals	48 hr ^{59}Fe incorp in femur	48 hr ^{59}Fe incorp in spleen
controls ♂ 0 8 ml glucose i p	5	0 762 ± 0 202	13 679 ± 6 115
db-cyclic AMP 6 μmol i p at 2 hrs intervals ♂	5	0 769 ± 0 335	12 850 ± 5 953
controls ♀ 0 8 ml glucose i p	5	0 934 ± 0 258	3 232 ± 2 044
db-cyclic AMP 6 μmol i.p at 2 hrs' intervals ♀	5	0 829 ± 0 405	5 219 ± 4 448

The values represent the mean of each group ± standard deviation

within comparable groups of treated mice and the controls

A standard preparation of human urinary erythropoietin was given alone and in combination with cyclic AMP to posthypoxic erythrocytotic mice to disclose, if possible, an enhancing effect of the cyclic AMP on the erythropoietin effect, but no such effect was seen

Theofyllin is a well known inhibitor of phosphodiesterase and is therefore, able to enhance the effect of cyclic AMP In spite of this theofyllin in up to sublethal doses in combination with cyclic AMP failed to show any effect on the erythropoiesis

DISCUSSION

The effect of cyclic AMP on the erythropoiesis has been investigated earlier by *Schooley & Mahlmann* (1971) who used carbon monoxide induced erythrocytotic mice as test animals The present investigations were initiated to reinvestigate this problem in the hope of elucidating the mechanisms of action The observed effect might be attributed to two obvious possibilities Cyclic AMP might stimulate the production of erythropoietin in the kidneys or cyclic AMP might act as a 'second messenger" to erythropoietin, the "first messenger" This mechanism is described for several other hormones (*Robinson et al* 1971) Accordingly, it was contemplated to use nephrectomized and normal mice in an attempt to elucidate which one of these possible mechanisms was involved

From the very beginning of the experiments, however, it became clear to us that the previously described effect on the erythropoiesis could not be demonstrated in our strain of mice (NMRI) under the experimental conditions used The doses of cyclic AMP were the same as those used by *Schooley & Mahlmann* (1971), and the experimental set-up differed mainly in the fact that *Schooley & Mahlmann* used mice in which the erythrocytosis was induced by carbon monoxide, while we used posthypoxic mice The dose schedules were varied and the route of administration was varied all with no demonstrable effect

A conceivable reason for the observed discrepancy might be that the erythropoiesis is more effectively suppressed in our mice and thus, that cyclic AMP, in Schooley & Mahlmann's experiments would act by enhancing the effect of the remaining slight amount of erythropoietin in the plasma Accordingly, we, gave a moderate dose of erythropoietin with and without the addition of cyclic AMP but found no enhancement of the erythropoietic stimulation In normal mice given cyclic AMP, any effect could not either be demonstrated

Consequently, we are forced to conclude that, in our NMRI strain of mice, the stimu

lating effect, if any, of cyclic AMP on the erythropoiesis is negligible

---

Aided by grants from the *Danish Cancer Society* and *Carl Schepler and Wife's Bequest*, the *Irma Foundation*

REFERENCES

Robison, G A, Nahas G G & Triner, L (Eds) Cyclic AMP and Cell Function Ann N Y Acad Sci *185* 1–556, 1971

Schooley, J C & Mahlmann, L. J Stimulation of Erythropoiesis in the Plethoric Mouse by Cyclic AMP and Its Inhibition by Antierythropoietin Proc Soc exp Biol (N Y) *137* 1289–1292 1971

Thorling, E B Dilution Anaemia. Absence of Compensatory Erythropoiesis in BALB/c Mice Developing Dilution Anaemia in the Course of Growth of a Transplanted Plasmocytoma Acta path microbiol scand Section A *78* 231–235, 1970

Documenta Geigy Wissenschaftliche Tabellen 6 Auflage, 1960 Herausgeber J R. Geigy A G Pharmazeutische Abteilung Redaktion *Konrad Diem*

Acta path microbiol scand Section A 81, 3, 233–240, 1973

THE PROLIFERATIVE ACTIVITY OF THE MYOCARDIAL TISSUE IN VARIOUS FORMS OF EXPERIMENTAL CARDIAC HYPERTROPHY

ARNE LJUNGQVIST and GUNNAR UNGE

The Departments of Pathology and Thoracic Medicine,
Karolinska sjukhuset, Stockholm Sweden

Various forms of experimental cardiac hypertrophy were investigated by light microscope autoradiography and measurements of tissue radioactivity after *in vivo* injections of ^{3}H thymidine in rats In the autoradiograms an attempt was made to distinguish between labelled vessel wall cells, connective tissue cells and heart muscle cells. A highly significant increase in the nuclear incorporation of ^{3}H thymidine was recorded in both ventricle walls of rats with cardiac hypertrophy induced by swimming exercise The increased incorporation was mainly confined to the nuclei of cells in the walls of the blood capillaries A considerably smaller increase in the incorporation into capillary wall cells was recorded in the left ventricle wall of rats with cardiac hypertrophy secondary to prolonged arterial hypertension and aortic stenosis of 1–2 weeks' duration. No increased incorporation of ^{3}H thymidine into the myocardial tissue was found in rats if their swimming exercise was followed by a period of rest, in rats with short term hypertension and in rats with aortic stenosis of 2 months duration. The observations suggest a significant neoformation of myocardial blood vessels during swimming exercise, whereas any neoformation of myocardial blood vessels in arterial hypertension and aortic stenosis appears to be of minor degree

In the hypertrophied heart, the diameter of the individual muscle cell is usually increased (*Karsner, Saphir & Todd* 1925) and muscle cell mitosis is infrequent (*Meerson* 1962, *Grove, Zak, Nair & Aschenbrenner* 1969, *Eisenstein & Wied* 1970) Consequently the heart enlargement is generally regarded as the result of an increase in volume of the individual muscle cells and not of an increase in their number (*Scotti* 1966) In agreement with this view the hypertrophied heart shows a proportionally larger increase in muscle mass than in DNA content (*Sumner & McIntosh* 1963, *Gluck, Talner, Stern, Gardner & Kulowich* 1964, *Grimm, Ryokubota & Whiteborn* 1966 and *Morkin & Ashford* 1968) The major portion of the increase in DNA is the result of an increased DNA synthesis in the interstitial tissue (*Morkin & Ashford* 1968) a minor degree of DNA synthesis may take place in the muscle cells with resulting polyploidia (*Grove, Nair & Zak* 1969 and *Grove et al* 1969)

Previous works did not determine the extent to which the various types of interstitial cells contribute to the production of DNA *in* the hypertrophied myocardium (*Morkin & Ashford* 1968 and *Eisenstein & Wied* 1970) Nor have any studies of the interstitial DNA synthesis activity in various forms of cardiac

Received 24.x.72 Accepted 16.xi.72

Requests for reprints should be addressed to *Arne Ljungqvist* M D Department of Pathology Karolinska sjukhuset S-104 01 Stockholm Sweden



Fig 1 Autoradiogram of left ventricle wall of hypertrophied rat heart (hypertension 3 months) showing one labelled smooth muscle cell nucleus *(arrow)* in the wall of a small artery × 900

hypertrophy been presented Since recent observations suggest a neoformation of myocardial blood vessels in certain forms of cardiac hypertrophy but not in others (*Ljungqvist & Unge* 1972), we have made an attempt to determine the DNA synthesis activity in various forms of cardiac hypertrophy and to differentiate between the activities in the vessel wall cells connective tissue cells and striated muscle cells

MATERIAL AND METHODS

Forty six *female Sprague-Dawley rats were used* The age of the rats at start of the experiments ranged between 2 5 and 5 5 months but within each group of rats (see below) the animals were of the same age at the time they were killed The rats were housed in cages 3–4 rats in each cage and were given a standard laboratory diet containing 0 4 per cent sodium chloride and tap water *ad libitum* Cardiac hypertrophy was induced by the production of renal hypertension, aortic stenosis and by swimming exercise (*Ljungqvist & Unge* 1972) The animals were grouped as follows

Group I Nine rats in which renal hypertension was produced by the application of an 0 15 mm wide silver clip on the left renal artery The blood pressures were measured with the tail pletysmographic method at daily (group I A) or weekly intervals (group I B) Hypertension developed within 2 6 weeks The animals were divided into two subgroups

I A Five animals which were killed within the first week of hypertension

I B Four animals which were killed 3 months after the development of hypertension

into three subgroups



Fig 2 Autoradiogram of left ventricle wall of hypertrophied rat heart (swimming exercise for 1 month) showing two labelled nuclei of capillary wall cells (*larger arrows*) Erythrocytes are seen in the capillary lumina (*smaller arrows*) × 2200

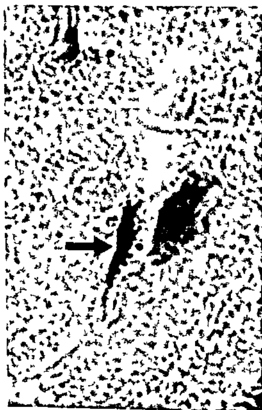


Fig 3 Autoradiogram of left ventricle wall of hypertrophied rat heart (swimming exercise for 3 months) showing one labelled connective tissue cell nucleus (*arrow*) × 2200

II A Four animals which were killed 5 days after the operation

II B Four animals which were killed 2 weeks after the operation

II C Four animals which were killed 2 months after the operation

Group III Sixteen animals were subjected to swimming exercise for 1 hour each day, 6 days per week They were divided into four subgroups

III A Four animals which were killed after the second week of exercise

III B Four animals which were killed after 4 weeks exercise

III C Four animals which were killed after 3 months exercise

III D Four animals which were killed after 3 months exercise followed by a resting period of 2 months

Group IV This group consisted of 4 normal rats the age of which was chosen to make them suitable as controls for the rats of groups I A, II C and III C

Group V This group consisted of 5 normal rats the age of which was chosen to make them suitable as controls for the rats of groups II A and B, and III A and B They also served as a control group for the slightly older rats in groups I B and III D

On the last day of each experimental period, the body weight of each rat was determined and 2 μCi ^{3}H thymidine/g body weight (specific activity 5 0 Ci/mM, Amersham, England) injected intraperitoneally The animals were killed by an overdosage of ether 24 hours after the injection of thymidine and the hearts were removed and weighed The heart/body weight ratios were calculated and multiplied by 1000 for practical reasons After fixation of the hearts in 10 per cent formaldehyde solution and paraffin embedding 4 μ thick transverse sections were taken from the ventricle portions of the heart and placed on glass slides The sections were covered with Kodak AR 10 film for autoradiography according to the stripping technique The films were exposed for 28 days at +4°C, developed in Kodak D 19 B and fixed in Kodak



Fig 4 Autoradiogram of left ventricle wall of hypertrophied rat heart (swimming exercise for 1 month) showing one labelled striated muscle cell nucleus (*arrow*) × 1350

TABLE 4 *The Tissue Radioactivity in Left Ventricle Wall after ³H Thymidine Injections in Rats with Various Forms of Cardiac Hypertrophy*

Group	Dur	c p m
I A	< 1 week	198 ± 143
I B	3 months	173 ± 25
II A	5 days	174 ± 17
II B	2 weeks	190 ± 14
II C	2 months	233 ± 114
III A	2 weeks	299 ± 14
III B	4 weeks	282 ± 37§
III C	3 months	313 ± 27†
III D	3 + 2 months	178 ± 13
IV		230 ± 9
V		196 ± 30

Group I = renal hypertension Group II = aortic stenosis Group III A C swimming exercise Group III D = swimming exercise followed by rest Groups IV and V = control rats Dur = duration of experimental situation c p m = counts per minute *The background activity measured on non injected material was* 35 ± 3 The figures are means ± SD

§ Significantly different from control group (0 001 < p < 0 01)

† Significantly different from control group (p < 0 001)

into a tissue is therefore widely used as a measure of its proliferative activity The degree of incorporation can be assessed after administration of radioactively labelled thymidine either by direct measurement of the tissue radioactivity or by calculation of the labelling index on autoradiograms The former method makes it possible to determine the quantity of thymidine incorporated into the tissue, but does not permit of an evaluation of the proportions of thymidine incorporated into the various tissue components The latter method can only give a semiquantitative information about the incorporation but provides means for an evaluation of the relative distribution of the thymidine in the various tissue components

In the present investigation the proliferative activity of the myocardial tissue components was determined both by measurements of tissue radioactivity and by calculations of the labelling indices following *in vivo* injections of ³H thymidine into Sprague Dawley rats Rats normally have a rapidly decreasing cellular proliferation in their hearts after birth and, at the age of 4 months the proliferative activity in the normal myocardial tissue is very low (*Enesco* & *LeBlond* 1962 *Petersen* & *Baserga* 1965, *Klinge* & *Stöcker* 1968 and *Sasaki*, *Morishita* & *Yamagata* 1970) The ages of the present animals at the time they were killed ranged between 3 5 and 8 5 months and the thymidine incorporation into their hearts should consequently be small Since certain age differences cannot be excluded even after 3 5 months, two control groups were used to cover the age range of the experimental groups One group of control rats was aged 5 5 months and the other 7 5 months when killed, the tissue radioactivities and total labelling indices of these two groups were however not significantly different

If compared with the control groups, it was found that the groups of rats that were killed immediately after terminated swimming exercise showed a highly significant increase in total labelling indices in their hearts An increase in total labelling index was recorded also in the left ventricle wall of rats in which aortic stenosis had been present for 2 weeks but this increase was of a minor degree it is in agreement however with the increase in interstitial cell labelling observed by *Morkin* & *Ashford* (1968) and *Grove et al* (1969) in hearts from rats with aortic stenosis of 1 and 2 weeks duration The importance of the elevation of the total labelling indices in the exercised rats was further evidenced by the fact that only the hearts of these rats showed an increase in tissue radioactivity which was statistically significant after 1 and 3 months of exercise On the basis of the observations made it can therefore be established that the myocardial tissue had experienced an increased proliferative activity during the swimming exercise whereas an unequivocal increase in proliferative activity could not be demonstrated in the myocardial tissue of rats with arterial hypertension and aortic stenosis.

The examination of the autoradiograms further showed that the increased incorpora

tion of thymidine into the myocardial tissue of the exercised rats was almost exclusively confined to the cells in the walls of the blood capillaries, indicating a neoformation of myocardial blood vessels in these rats. The lowering of the thymidine incorporation to normal levels during the period of rest following terminated exercise suggests that the swimming was the direct stimulus to the neoformation of blood vessels These findings are in good agreement with our previously reported observations on stereo-micro-angiograms according to which swimming exercise was seen to lead to an increase in density of the myocardial vasculature (*Ljungqvist* & *Unge* 1972), this vasculature was remodelled, but not further increased, during the resting period following termination of the exercise

The magnitude of the increase in thymidine incorporation into the myocardial capillary walls of the exercised rats, and the fact that the hearts of these rats show microangiographically visible increased vasculatures, strongly suggest that the neoformation of myocardial blood vessels during exercise is of importance and that it may provide a basis for an adequate oxygenation of the enlarging myocardial musculature In cardiac hypertrophy secondary to arterial hypertension and aortic stenosis, on the other hand, the increase in thymidine incorporation into the capillary walls was of minor degree and not sufficient to lead to any increase in total labelling index (except after 2 weeks aortic stenosis) and tissue radioactivity, suggesting that neoformation of myocardial capillaries was insignificant in these rats Even a significant capillary neoformation would however have escaped detection by the present methods if it had occurred before the injections of ^{3}H thymidine It is not probable that this should occur in the hearts of rats with arterial hypertension and aortic stenosis in view of the fact that such hearts did not show any increased vascular density in the micro-angiograms (*Ljungqvist* & *Unge* 1972)

The fact that swimming exercise appears to be a far more important stimulus for myocardial capillary neoformation than aortic stenosis and arterial hypertension becomes even more clear if the labelling indices of the capillary wall cells in the exercised rats are compared with those of the rats with aortic stenosis and arterial hypertension in which the stress situation had been acting for comparable periods of times (Table 2) In this respect group III A can be compared with II B (period of cardiac stress 2 weeks) and group III C with I B (period of cardiac stress 3 months), whereas it applies to group III B that a comparable group with aortic stenosis or hypertension is not available Thus, it appears that the capillary wall labelling index is significantly higher in rats that had been swimming for 2 weeks (group III A) than in rats in which aortic stenosis had been present for the same period of time (group II B, $0.001 < p < 0.01$), these groups of rats are also quite comparable with respect to age (Table 1) The capillary wall labelling index of rats that had been swimming for 3 months (group III C) was apparently significantly higher than that in rats which had been hypertensive for a comparable period of time (group I B $p < 0.001$), admittedly, the former rats were younger, but still sufficiently old to be regarded as full grown (Table 1)

Labelled nuclei judged to belong to connective tissue cells were encountered in all groups of rats but they were less numerous by far than labelled capillary wall cells The distribution of the labelling indices of the two cell types in the various groups of rats was similar, and it is possible that some cells judged as connective tissue cells were in fact located in the capillary walls the lumen of the capillary being out of the plane of sectioning or collapsed

The incorporation of thymidine into nuclei of striated heart muscle cells is extremely low both in the normal and in the hypertrophied heart (*Meerson Alekhina, Aleksandrov* & *Babardjan* 1968 *Morkin* & *Ashford* 1968 *Grove et al* 1969, *Sasaki et al* 1970, *Sasaki, Morishita, Ichikawa* & *Yamagata* 1970 and *Neffgen* & *Korecky* 1972) This was verified in the present investigation and lends further support to the commonly accepted theory that

cardiac hypertrophy is not the result of an increase in the number of heart muscle cells

Increased thymidine incorporation into cells in the walls of arteries and arterioles was recorded only in the hypertensive rats This is in good agreement with the cellular hyperplasia seen in the arterial walls of hypertensive individuals and the increased thickness of the walls of the myocardial arteries observed in hypertensive animals (*Crane* & *Dutta* 1963, *Crane* & *Ingle* 1964 and *Ljungqvist* & *Unge* 1972)

This study was supported by the *Swedish Medical Research Council* (Project No B72 12X-716-07A), the *Swedish National Association against Heart and Chest Diseases* and the *Research Funds of Karolinska Institutet*

REFERENCES

1 *Cleaver, J E* Thymidine metabolism and cell kinetics North Holland Research Monographs Frontiers of Biology, vol 6, Amsterdam, North Holland publishing Co, 1967

2 *Crane, W A J* & *Dutta, L P* The utilisation of tritiated thymidine for desoxyribonucleic acid synthesis by the lesions of experimental hyper tension in rats J Path Bact *86* 83-97, 1963

3 *Crane, W A J* & *Ingle, D J* Tritiated thymidine uptake in rat hypertension Arch of Pathol *78* 209-221, 1964

4 *Eisenstein, R* & *Wied, G* Myocardial DNA and protein in maturing and hypertrophied human hearts Proc Soc Exptl Biol Med *133* 176 179 1970

5 *Enesco, M* & *LeBlond, C P* Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. J Embryol Exp Morphol *10* 530-562 1962

6 *Gluck, L*, *Talner, H S*, *Stern, H*, *Gardner, T H* & *Kulovich, M V* Experimental cardiac hypertrophy concentrations of RNA in the ventricles Science *144* 1244-1245 1964

7 *Grimm A F*, *Ryo Kubota, B S* & *Whiteborn W V* Ventricular nucleic acid and protein levels with myocardial growth and hypertrophy Circul Res *19* 552 558 1966

8 *Grove, D*, *Nair, K G* & *Zak, R* Biochemical correlates of cardiac hypertrophy III Changes in DNA content, The relative contributions of polyploidy and mitotic activity Circul Res. *25* 463-471, 1969

9 *Grove, D*, *Zak, R*, *Nair, K G* & *Aschenbrenner, V* Biochemical correlates of cardiac hypertrophy IV Observations on the cellular organization of growth during myocardial hypertrophy in the rat Circul Res *25* 473-485 1969

10 *Karsner, H*, *Saphir, O* & *Todd, W T* The state of the cardiac muscle in hypertrophy and atrophy Am J Pathol *1* 351-371, 1925

11 *Klinge, O* & *Stöcker, E* Die DNS Synthese im Rattenherzen als Funktion des Lebensalter Autoradiographische Untersuchungen mit ³H Thymidine Experientia *24* 167-168, 1968

12 *Ljungqvist, A* & *Unge, G* The finer intra myocardial vasculature in various forms of experimental cardiac hypertrophy Acta path microbiol scand Sect A *80* 329-340, 1972

13 *Meerson, F Z* Compensatory hyperfunction of the heart and cardiac insufficiency Circul Res *10* 250-258, 1962

14 *Meerson, F Z*, *Alekhina, G M*, *Aleksandrov, P N* & *Bazardjan, A G* Dynamics of nucleic acid and protein synthesis of the myocardium in compensatory hyperfunction and hypertrophy of the heart Am J Cardiol *22* 337-348 1968

15 *Morkin, E* & *Ashford, T P* Myocardial DNA synthesis in experimental cardiac hypertrophy Am J Physiol *215* 1409 1413 1968

16 *Neffgen, J F* & *Korecky, B* Cellular hyperplasia and hypertrophy in cardiomegalies induced by anemia in young and adult rats. Circul Res *30* 104-113, 1972

17 *Petersen, R O* & *Baserga, R* Nucleic acid and protein synthesis in cardiac muscle of growing and adult mice Exptl Cell Res *40* 340-352, 1965

18 *Sasaki, R*, *Morishita, T* & *Yamagata, S* Autoradiographic studies on heart muscle cells in normal rats Tohoku J Exp Med *100* 1-13, 1970

19 *Sasaki, R*, *Morishita, T*, *Ichikawa S* & *Yamagata, S* Autoradiographic studies and mitosis of heart muscle cells in experimental cardiac hypertrophy Tohoku J Exp Med. *102* 159-167 1970

20 *Scotti, T M* Heart In Pathology ed. by W A D Andersson St Louis Mosby vol. 1, p 526 1966

21 *Sumner, R G* & *McIntosh H D* Nucleic acid studies in experimental cardiomegaly Circul Res. *12* 170-175 1963

Acta path microbiol scand Section A 81, 3, 241–246, 1973

INCREASED CELLULAR REACTION TO DAMAGE CAUSED BY ANGIOTENSIN IN ARTERIOLES OF NORMAL RECIPIENT RATS AFTER TRANSFER OF LYMPHOCYTES FROM HYPERTENSIVE RATS

ULRIK GERNER SVENDSEN

The University Institute for Experimental Medicine
(Head Jens Bing, M D) Copenhagen, Denmark

The presence of an immunological factor in vascular disease in renal hypertensive rats is demonstrated by showing, that transfer of thoracic duct cells from these rats to normal syngeneic recipients is followed by a change in the reactivity to injections of angiotensin with the result that the normal recipients react with a secondary cellular response in the damaged arterioles

The existence of an immunological factor in the pathogenesis of experimental hypertensive vascular disease has been discussed for many years *Otha et al* (1959) found deposits of gamma globulin in the vascular lesions in experimental necrotizing arteritis in rats, and *Okuda & Grollman* (1967) demonstrated that the hypertension induced by infarction of the kidney in rats was transferred by viable lymph node cells into rats which were rendered immunologically tolerant by neonatal pretreatment with normal rat spleen cells It has further been shown by *Gardner et al* (1970) that the frequency of arteriolar damage was decreased in hypertensive rats treated with immuno-suppressive drugs

The existence of an immunological factor in hypertensive vascular disease has recently been supported in studies by *Olsen* (1971) who showed that the cellular reaction ("primary response") in the arterioles of rats with acute hypertension caused by series of injections of angiotensin, is changed both in degree and duration ("secondary response") if the injections are repeated after at least one week In further experiments he showed that the more pronounced and prolonged secondary response would occur already after the first series of angiotensin injections if the rats had been pretreated with injections of thoracic duct lymphocytes from donor rats which had received repeated series of angiotensin injections at intervals of about one week

The aim of the present study has been to investigate whether rats with *chronic renal hypertension* develop cellular immunity against their own vascular walls in such a way that transfer of thoracic duct cells from these

Received 20 xi 72 Accepted 5 xii 72

Requests for reprints should be addressed to *Ulrik Gerner Svendsen*, University Institute for Experimental Medicine, 71 Norre Alle, 2100 Copenhagen Ø Denmark

animals to syngeneic normal recipients will show that they react with a secondary response if exposed to marked acute increases in the blood pressure, caused by repeated injections of angiotensin throughout 5 hours. As a basis for the research, it was investigated whether it might be possible to reproduce the secondary cellular response in rats, as described by *Olsen* (1971).

MATERIAL AND METHODS

Animals. Inbred female SPF hood rats and SPF Wistar rats weighing 170–180 grams from Mollegaard Hansens Avlslaboratorier A/S.

Anaesthesia. A solution of penthymal sodium 25 mg/ml injected intraperitoneally in a dose of 125 mg per kg. b.w. A larger dose was used when rats were killed.

Colloidal carbon particles specially produced for experimental use (Gunther-Wagner Pelikan Werke, Hannover, Germany C 11/1431 a).

Angiotensin II-amid. Hypertension CIBA dissolved in physiological saline.

The Experimental Technique

Group I. (Primary response) 8 Wistar and 4 hood rats were treated as described by *Olsen* (1971). After complete anaesthesia had been induced, a catheter of polyethylene was placed in the jugular vein and ½-1 µg of angiotensin injected intravenously every 5th minute for 5 hours. At the termination of the injections of angiotensin, 0.1 ml of colloidal carbon particles was injected intravenously and the blood pressure was kept at hypertensive values (i.e. more than 145 mm Hg.) by continuous injections of angiotensin (2 µgram/minute) for 10 minutes. The rats were killed 24, 48, 72 and 96 hours after the termination of the hypertensive period. The small intestine was fixed in buffered formalin. Mesenteric arterioles and arterioles situated in the submucosa of the small intestine with colloidal carbon particles deposited in the walls were isolated, embedded in paraffin, cut in 5 micron thick serial sections and stained with PAS (per iod acid Schiff). From each rat, all arterioles with typical deposits in the arteriolar wall *(Giese* 1966) were isolated. After em-

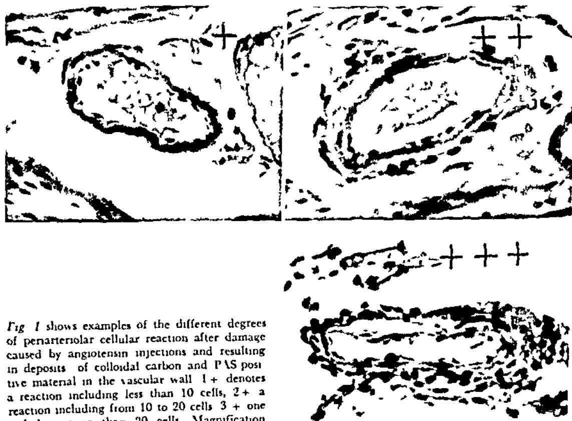


Fig. 1 shows examples of the different degrees of periarteriolar cellular reaction after damage caused by angiotensin injections and resulting in deposits of colloidal carbon and PAS positive material in the vascular wall. 1+ denotes a reaction including less than 10 cells, 2+ a reaction including from 10 to 20 cells. 3+ one including more than 20 cells. Magnification 480 ×. PAS staining.

bedding in paraffin, 10 arterioles were selected at random and cut in serial sections From each arteriole, about 250 serial sections were studied under the microscope In sections with cellular infiltrations around hypertensive, damaged arterioles (i.e with colloidal carbon and PAS positive material deposited in the vascular wall), the intensity of the cellular reaction was determined following a scale from 1+ to 3+, where 1+ means a reaction with 10, 2+ from 11–20 and 3+ more than 20 cells in close connection to the vessel (Fig 1) The reaction was only counted if the degree of cellular infiltrations was found in an extent which was more than 25 μ, i.e 5 consecutive serial sections, of an arteriole, and the most pronounced reaction was used

Group II (Secondary response) 8 Wistar and 8 hood rats were treated twice, at intervals of 7 days, with angiotensin as described under group I 24 48 72 and 96 hours after the termination of the second angiotensin treatment, the animals were killed and further treated as described under group I

Group III (The cellular reaction in animals after transfer of thoracic duct cells from renal hypertensive or normal rats) Thirty three hood rats were divided into 2 groups of 13 and 20 animals and a 0 20 mm silver clip was placed on their right renal artery The first group consisted of 13 animals whose blood pressure was measured once to twice weekly for 3 months by the tail cuff method (*Williams et al.* 1939) At the end of this period, the animals presenting the most marked hypertension were selected and used as donors In the morning, a polyethylene catheter was placed in the abdominal part of the thoracic duct in these donor rats and the lymph was collected at 2–4° C for 1 3 days The rats were able to eat and drink freely throughout this period Lymph portions collected during the night were used Each portion consisted of 120–180 million viable cells estimated in phase contrast microscope The cells were washed 3 times in Minimum essential medium (Flow lab, Scotland), resuspended in 1 ml of medium and injected into the recipients. Angiotensin treatment was started 30 minutes later The further treatment was as described under group I The *control animals* were hood rats which were treated as the experimental animals except that they received thoracic duct cells from *normal* syngeneic rats After the experiments, the *donor rats* were killed and the heart, pancreas and the untouched kidney were fixed in formalin, embedded in paraffin and cut in sections which were stained by PAS and Van Gieson Hansens methods The heart weight and haematocrit were determined.

The second group investigated about 4 months after the first consisted of 20 hood rats which were treated in the same way as the first group except that all the animals were killed 72 hours after the 5 hours repeated angiotensin injections.

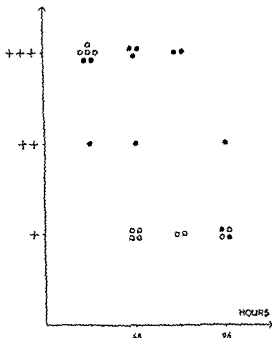


Fig 2 shows the marked difference in the degree of periarteriolar cellular reaction in the primary (O) and the secondary (●) response to a single and a repeated damage (caused by angiotensin injections), respectively While the primary response has its maximum only 24 hours after the damage, such maximal values are also found after 48 and 72 hours in rats with the secondary response The degree as measured by the method shown in fig 1, is plotted along the ordinate while the time in hours after the damage is plotted along the abscissa.

RESULTS

Group I In the experiments performed with a view to investigating the *primary* cellular reaction after acute angiotensin induced vascular damage, all the animals survived the treatment and there were typical (*Giese* 1966) carbon deposits in the arterioles. The results are illustrated in Fig 2 which shows that the cellular infiltration increases to a maximum after 24 hours At 48 hours it had decreased to normal value

Group II In the experiments performed with a view to investigating the *secundary* cellular reaction after acute angiotensin induced vascular damage, 2 animals died after the treatment and there were no carbon deposits in 2 animals The results obtained

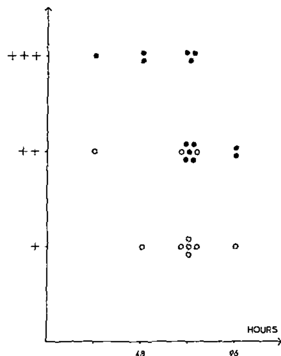


Fig 3 shows the marked difference in the periarteriolar cellular reaction to a single series of angiotensin injections in recipient rats pretreated with thoracic duct cells from hypertensive (●) and normal (○) donors Ordinate and abscissa as in Fig 2

in the remaining experiments are illustrated in Fig 2 which shows that the cellular infiltration increases to a maximum after 24 hours in these cases the same maximum reaction was maintained also after 48 and 72 hours At 96 hours it had decreased to normal

Group III In the experiments performed with a view to investigating *the cellular reactions in angiotensin induced vascular damage after transfer of thoracic duct cells from hypertensive rats* all the donors were in good health and non of these had suffered a hypertensive crisis in the 14 days prior to the operation The blood pressure measured in the afternoon in these hypertensive donor rats ranged between 170 and 240 mm Hg They presented hypertrophy of the heart the relative heart weigth ranging at 0 42 % against 0 31 % (heart weight x 100/body weight) in the normotensive control rats Hypertensive vascular disease with moderate PAS positivity and moderate perivascular connective tissue proliferation was found in all hypertensive donors The haematocrit value was about 40 (range 35 46) in hypertensive as well as in control rats

Four recipients in which the acute angiotensin hypertensive treatment failed to produce vascular lesions of a degree sufficient to bring about the typical deposit of colloidal carbon were not used for further investigations The other 7 donors gave cell portions to recipients in which the typical carbon deposits in the arterioles occurred after treatment with angiotensin The results of the microscopic investigations of the hypertensive, damaged mesenterial arterioles from the recipients are illustrated in Fig 3 and 4 which show the difference in the response, responses of recipients pretreated with cells from renal hypertensive donors were markedly higher and more prolonged than responses of those receiving cells from normal donors The difference in the mean reactions in all the investigated vessels from all the animals which were killed 72 hours after the acute hypertensive period was significantly different in the experimental animals and in the control animals (Wilcoxon's rank sum test $p<0.01$)

DISCUSSION

A Primary and secondary response The results obtained in the experiments on the primary and secondary cellular response around arterioles damaged by repeated injections of angiotensin are shown in Fig 2 and confirm the results obtained by *Olsen* (1970) according to which an angiotensin hypertensive episode is followed by an inflammatory cellular reaction with maximal intensity after 24 hours Forty eight and 72 hours after the injections the predominant mononuclear cellular reaction had decreased to normal (primary response) If the treatment was repeated 8 days later the duration of the cellular reaction would be prolonged up to 72 hours indicating that the animals after the first hypertensive episode

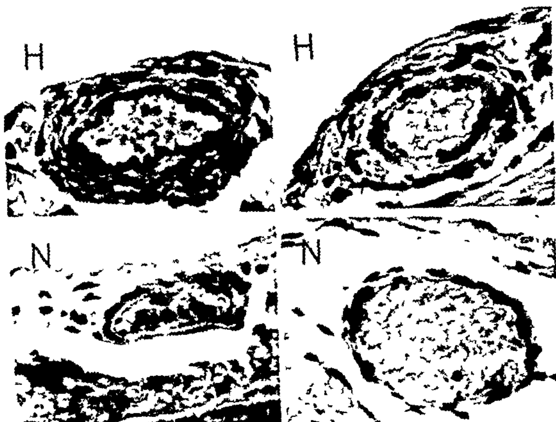


Fig 4 Mesenteric arterioles with deposits of colloidal carbon and PAS positive material in the walls from rats pretreated with thoracic duct cells from renal hypertensive donors (H) and from normal donors (N) respectively and subsequently treated with angiotensin and killed 72 hours after the termination of the hypertensive period After 48 and 72 hours there is a marked mononuclear cellular reaction around the arterioles from the animals pretreated with thoracic duct cells from hypertensive donors but only a slight reaction in those pretreated with cells from normal donors Magnification 480 X PAS staining

are immunized against their own vascular walls The secondary response was dominated by mononuclear cells from the onset to the termination of the reaction The cellular reaction in the bred rats was identical but too few animals were investigated to give a conclusive result regarding any species difference in the reaction

B Secondary responce after transfer of thoracic duct cells from hypertensive rats The results obtained in the experiments with renal hypertensive rats show that the latter develop an immunity against their own vascular walls an immunity which can be transferred by washed thoracic duct cells These results are shown in Fig 3 and 4 which show that while the cellular reaction in the control animals is similar to the primary response the cellular reaction in the rats which were pretreated with cells from hypertensive donors is similar to the secondary response The importance of the immunological factor in the pathogenesis of the hypertension or the hypertensive vascular disease is so far unknown

The author is grateful to *H E Nielsen* M D The University Institute for Experimental Immunology Copenhagen for valuable advice and to Miss *Lisbeth Olsen* for technical assistance This work was supported by grants from the *Danish Medical Research Council* Kobmand i Odense *Johann og Hanne Weimann* foundation and *Thomas Bartholin s* foundation The Hypertensin® was kindly supplied by Ciba Geigy Copenhagen

REFERENCES

1 *Gardner, D L, Quagliata, F, Drossman, M, Kalish, M & Schimmer, B* Attempted prevention of arteriolar lesions in accelerated rat hypertension by immunosuppression Br J Exp Path *51* 242–252, 1970

2 *Giese, J* The pathogenesis of hypertensive vascular disease Munksgaard, Copenhagen, 1971 p 172

3 *Ohta, T, Cohen, S, Singer, E J & Strauss L* Demonstration of gamma globulin in vascular lesions of experimental necrotizing arteritis in the rat. Proc. soc. exp biol (N Y) *102* 187–189 1959

4 *Okuda, T & Grollman, A* Passive transfer of autoimmune induced hypertension in the rat by lymph node cells Texas rep Biol Med *25* 257–264 1967

5 *Olsen, F* Inflammatory cellular reaction in hypertensive vascular disease Munksgaard Copenhagen, 1971

6 *Williams, J R, Harrison, T R & Grollman, A* A simple method for determining the systolic blood pressure of the unanaesthetized rat J Clin Invest *18* 373–374, 1939

Acta path. microbiol. scand Section A, 81, 3, 247–253 1973

CAUSE OF THE PROLONGED PRESSOR ACTION OF RENIN IN NEPHRECTOMIZED RATS

Elucidated by Means of Anti Angiotensin II and of Angiotensin Inhibitor (1 Sar-8 Ala Angiotensin II)

JENS BING and KNUD NIELSEN

The University Institute for Experimental Medicine,
Norre Alle 71, 2100 Copenhagen Ø

In nephrectomized rats both the primary and the prolonged pressor response to exogenous renin the duration of which markedly exceeds the rate of disappearance of renin activity from plasma, is shown to be due to the continued action of renin the blood pressure being totally or subtotally normalized by infusion of an angiotensin inhibitor A marked but somewhat less pronounced decrease in the blood pressure is caused by injection of anti angiotensin II An accumulation of renin in the arterioles may account for these results. The height of the pressor response to renin is identical in anaesthetized and conscious normal rats but the length is markedly shorter in anaesthetized than in conscious animals.

The prolonged pressor action of renin in nephrectomized animals was observed by *Tigerstedt & Bergman* (1898) and has been confirmed by all later observations (see *Page & McCubbin* (1968) and *Lee* (1969)) The fact that the pressor effect of renin in bineph rectomized animals is prolonged by several hours (see Fig 2) has given rise to studies concerning the disappearance of injected ren in from the blood of normal and nephrectomized animals In dogs *Houssay et al* (1942) found that the disappearance of renin from the blood increased from less than 30 minutes up to approximately one hour in recently nephrectomized animals and up to 2½ hours in 48-hour nephrectomized animals In later studies by *Assaykeen et al* (1968) and *Schneider et al* (1968) the half life of renin was found to be approximately 45 80 minutes while *Basso & Taquini* (1970) found it to cover 4 hours.

The half life of renin has been found to be much shorter in *binephrectomized rats Schaechtelin et al* (1964) found that the prolonged pressor response to renin in the 20 hour nephrectomized rat was not connect ed with a prolonged persistence of renin in the blood from which it disappeared within one hour *Peters Haefeli* (1971) found a similar rate of disappearance, as shown by the curves with broken lines reproduced in figs 1 and 2 in the present paper

The aim of the present paper has been to elucidate the part played by the renin system in the *prolonged* postnephrectomy response to renin in a study of the influence exerted by anti angiotensin II and by a competitive angiotensin II inhibitor (1-Sar 8-Ala Angiotensin II) which completely blocks the pressor action of angiotensin by interfering with an-

Received 1 xii 72 Accepted 1 xii 72

giotensin on its vascular receptor site (*Pals et al* 1971) By way of comparison, studies on the effect of this inhibitor on the elevated blood pressure produced by continuous infusion of angiotensin were performed The paper includes a comparison of the pressor effect of renin in conscious or amobarbital anaesthetized normal animals and in nephrectomized rats

MATERIAL AND METHODS

Female Wistar S P F rats weighing approximately 200 g were anaesthetized either with 100 mg/kg b w of amobarbital (amytal) or with a short term ether anaesthesia During anaesthesia one carotid artery was *cannulated for the recording of blood pressure* using a Fybjærg Hansen transducer and a Servogor 511 recorder For the determination of the blood pressure in *conscious* rats, catheters were chronically implanted according to 1) the method of *Popovic* and *Popovic* (1960) by which one catheter is inserted via the carotid artery into the aorta and *another via the jugular vein into the superior cava* or to 2) a method including insertion of polythene PP 50 catheters about 5 10 mm into the carotid artery and jugular vein The artery catheters were filled with a solution of 1000 3000 I U of heparin per ml of physiological saline solution while a solution containing 100 I U herapin per ml was used for the vein catheters. The latter method was also used for the studies on *anaesthetized* rats. The reason why the *Popovic* method was used only in part of the studies on conscious rats was that technical difficulties encountered in some of the experiments *prevented the unobstructed passage through*

preparation was an immune plasma from a rabbit (K64E) with a neutralization capacity of 15 μg and an association constant of 2×10^{10} (*Bing* and *Poulsen* 1970) the dose being 300 μl a similar dose of plasma from a non immunized normal rabbit (K65E) served as control The *angiotensin inhibitor* was the 1 Sar 8 Ala *angiotensin II* (Sar Arg Val Tyr Val His Pro-Ala) from the Norwich Pharm Corp New York previously used by *Pals et al* (1971) One mg of the inhibitor was dissolved in 10 ml of an 0 9 per cent NaCl solution which was divided into 5 portions and kept frozen at −20° C in siliconized glass tubes The infusion *was given by means* of a Braun perfusor with a speed of 1 14 ml per hour resulting in a dose of about 2 μg/min/rat (≈ 10 μg/kg/min) In some *cases, the speed and thus also the dose* were doubled or, a 5 times higher concentration, resulting in a dose of 50 μg/kg/min was given.

RESULTS

1 *Pressor Effect of Renin on Anaesthetized or Conscious Normal and Nephrectomized Rats*

In *amytal anaesthetized normal rats* (17 tests on 10 rats) receiving 10 20 m G U renin, there was a pressor response with a height from 10 to 50 mm Hg and a lenght of about 8 (range 6-20) minutes, the response being dose dependent in most cases When the dose was 20 m G U the mean height was about 40 mm Hg (Fig 1) In 2 rats, the blood pressure did not return to the start level, but ended at a 15 20 mm higher value

In *conscious normal rats* (19 tests on 10 rats), the majority of which received 20 m G U renin, the height of the pressor response was about the same as that in the anaesthetized animals, while the response in most cases was more prolonged the blood pressure not returning to the start level until after about 20 30 minutes The difference between the

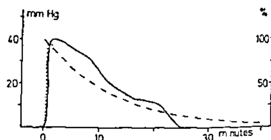


Fig 1 shows that while the height of the *pressor response to intravenous injection of 20 m G U* renin is the same in concious (——) and amytal anaesthetized ( ) *normal rats* the length of the response is much shorter in the anaesthetized than *in the conscious rats By way of comparison* the broken line ( ) shows the rate of disappearance of injected renin in anaesthetized normal rats as observed by *Peters Haefeli* (1971) The increase in blood pressure in mm Hg above the pre renin injection level *and the percentual rate of disappearance* of renin are plotted along the ordinate The time in minutes after the renin injection is plotted along the abscissa

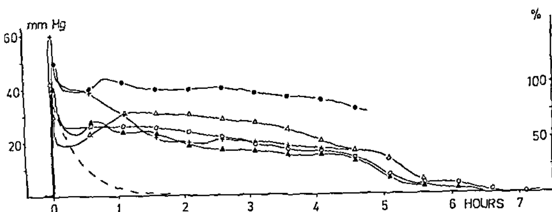


Fig 2 shows that the *pressor response to 20 m G U renin in five 24 hour nephrectomized conscious rats* is markedly prolonged and that the blood pressure level at start is not reached until after well over 5 7 hours By way of comparison, the broken line shows that the renin activity in plasma of nephrectomized rats injected with rat renin has almost disappeared within one hour, the values are those originally published by *Peters Haefeli* (1971) The increase in blood pressure, in mm Hg above the pre renin injection level, and the percentual rate of disappearance are plotted along the ordinate The time in hours after the renin injection is plotted along the abscissa

pressor effect of renin in conscious and amytal anaesthetized rats is shown in Fig 1, which further shows the rate of disappearance of renin activity from plasma, as observed by *Peters-Haefeli (1971)*

The pressor response to 20 milli G U renin in 4 *anaesthetized* animals and in 15 *conscious,* about *24 hour-nephrectomized, rats* did not differ significantly in height from that of normal rats, but presented the wellknown *prolongation, the blood pressure remaining* above the start level for hours, as shown in Fig 2 in which the broken line by way of comparison, shows the values obtained by *Peters-Haefeli* (1971) for the rate of disappearance of renin activity from plasma of nephrectomized rats injected with renin

2 Effect of Anti-Angiotensin II on the Prolonged Pressor Effect of Renin in Nephrectomized Rats

The effect of anti-angiotensin was studied in 1 anaesthetized animal and in 8 conscious rats which, approximately 24 hours after binephrectomy, responded to intravenous injection of 20 m G U renin with an approximate 40 mm high pressor response The *injections of anti angiotensin,* which were given from a few minutes to 3 hours after the renin-injection, *resulted in all experiments in a depression of the blood pressure which, however, still remained markedly above the pre-renin-injection level,* as shown by the examples given in Fig 3 which show how the prolonged pressor effect of 20 m G U renin given at time 0 is modified by injection of anti angiotensin, which in 3A and B is given a few minutes after the injection of renin, and in 3C and D *about 3 hours after such an injection* The reason why the blood pressure only partially returns to the start level is apparently not that the dose of anti-angiotensin was to small, as a second dose (Fig 3C) given 20 minutes after the first does not bring about any significant change, *the latter is in agreement with* our previous findings (*Bing & Poulsen,* 1968 and 1970) The effect of the anti-angiotensin doses given was further shown in studies of the sensitivity to synthetic angiotensin, which decreased to about 5 per cent of values found in the same animal before the injection of immune plasma Such decreased sensitivity was also found after the blood pressure had returned to the pre anti angiotensin level in the cases where the hypotensive effect of anti-angiotensin II was merely of 10-15 min-

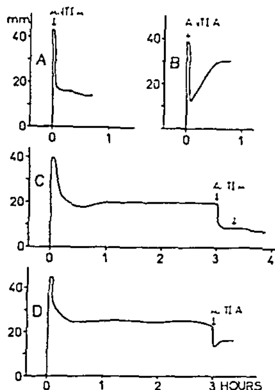


Fig 3 shows *the effect of anti-angiotensin (Anti A) on the prolonged pressor response* to 20 m G U renin *in conscious nephrectomized rats in which* the anti angiotensin II was given 1½ (A) and 2½ (B) minutes or 3 hours (C and D) after the renin injection. In one experiment (C) a second dose of anti angiotensin did not bring about a further significant decrease in blood pressure The ordinate and abscissa indicate increases in blood pressure in mm Hg above the pre renin injection level and time, respectively (cf Fig 2)

utes' duration Injection of the same dose of normal rabbit plasma gave either no response or a 3 10 mm depressor response of only 1 3 minutes' duration

3 Effect of the Angiotensin-Inhibitor on the Prolonged Pressor Effect of Renin in Nephrectomized Rats

The effect of the angiotensin inhibitor 1 Sar 8 Ala angiotensin II was studied in 13 experiments on 10 approximately 24 hour nephrectomized conscious rats which responded to 20 m G U renin with an approximate 35 mm high pressor response The continuous infusion of angiotensin inhibitor, which in most cases was given in doses of 10 μg/min/kg (about 2 μg/min/rat), was started from 10 min to 2¼ hours after the renin injection and continued for about 30 minutes (range 16-46 min), in some cases the dose was increased up to 20 μg/min/kg during the experiment The continuous *infusion of angiotensin inhibitor resulted in all experiments in a marked decrease in blood pressure* which in 2 experiments reached the pre renin injection level, as shown in Fig 4B and C, while a subtotal (Fig 4A), or only partial, return to the start level (Fig 4D) is shown in the other examples After the maximum lowering of the blood pressure had been reached, this value was in all but one experiment kept at this low level as long as the infusion was continued.

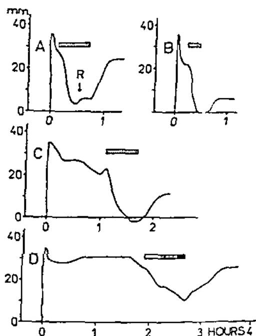


Fig 4 shows *the effect of angiotensin inhibitor on the prolonged pressor response* to 20 m G U renin in nephrectomized rats in which the continuous infusion of the inhibitor was started 10 (A) and 14 (B) minutes or about 2 hours (C and D) after the renin injection The dose of the inhibitor was in the range from 5 (marked ▭) to 10 (▭) to 20 (▭) or in one case 50 (■) μg/kg/min [1] In A a second dose of renin (marked R) was given during the infusion The ordinate and abscissa indicate increases in blood pressure in mm Hg above the pre renin injection level and time (cf Fig 2)

The rate at which the maximum depression of blood pressure was reached varied somewhat (range 3 23 minutes), as seen in Fig 4A-D which further show how the blood pressure increased again after discontinuation of the infusion In some cases it might even reach the value observed at the start of the infusion. In 3 cases in which the dose was changed from 10 to 20 µg/min/kg, and in one in which it was changed from 20 to 10, these changes did not influence the blood pressure level In yet another case (Fig 4D) in which the dose was changed from 5 to 10 to 50 µg there was, however, a further small decrease in blood pressure after each change The sensitivity to intravenously injected angiotensin and renin in doses which had given a marked response prior to the infusion of inhibitor, was abolished during the infusion, it was also decreased 30 60 min after its discontinuation

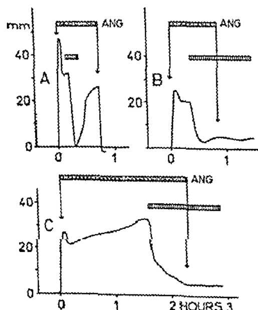


Fig 5 shows the effect of the inhibitor on the prolonged pressor effect caused by continuous infusion of 25 (in A) or 48 (B C) ng angiotensin/rat/min ¹ (marked [XXX]) The inhibitor was given in doses of 10 ([   ]) µg/kg/min (1 2 µg/rat/min) at intervals of from 9 min to 1¼ hours after the start of angiotensin infusion. The ordinate and abscissa indicate blood pressure increase in mm and time (cf Fig 4)

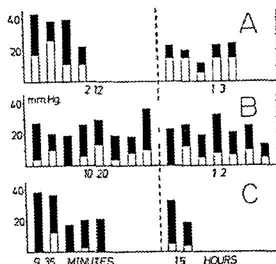


Fig 6 shows a comparison between the effect of anti angiotensin II (A) and angiotensin inhibitor treatment (B) of the prolonged pressor response to renin in nephrectomized rats with that of the inhibitor on angiotensin induced high blood pressure (C) The figure includes all experiments performed Each experiment is represented by a column The sum of the white and black part of each column indicates the blood pressure increase, in mm Hg, above the pre injection level of renin (A and B) or angiotensin (C) at the time at which the treatment *with anti-angiotensin (A) or inhibitor (B and C)* was started The hypotensive effect of the treatment is indicated by the height of the black column, the top of the white column thus marking the number of mm by which the blood pressure still exceeded the pre-renin (or angiotensin) injection level at the maximum effect The interval between 1) the injection of renin or start of infusion of angiotensin and 2) the start of the treatment is plotted along the abscissa.

4 Effect of Angiotensin Inhibitor on the Elevated Blood Pressure Caused by Continuous Infusion of Angiotensin II

As a control to the preceding experiments, the effect of the inhibitor on the elevated blood pressure in rats receiving continuous infusion of angiotensin was studied in experiments on 6 rats among which two, one normal and one nephrectomized, were anaesthetized, while four 24 hour nephrectomized animals were conscious Neither anaesthesia nor nephrectomy exerted any influence on the results obtained. As the rats differed in their sensitivity to injection of 5 ng of angio-

tensin the dose of continuous infusion of angiotensin varied from 125 240 ng/kg/min [1] (25 48 ng per min per rat) by which doses a blood pressure level approximately 20 35 mm above the pre infusion level was obtained This difference in dosage of angiotensin played *no role when a dose of 5 10 μg of the inhibitor/kg/min* was used The continuous *infusion of inhibitor* was started from 9 min to 1½ hours after the start of the angiotensin infusion and continued for from 13 minutes up to 1¼ hours *It resulted in all cases in a marked decrease in blood pressure* which in 3 rats reached the pre inhibitor infusion level and in 2 came close to it while the blood pressure in the last case remained about 12 mm above this level The rate at which the lowest value was reached varied somewhat (range 7 40 minutes) as seen in Fig 5 A C which further show the effect of discontinuation of infusion of angiotensin or (and) inhibitor

5 Comparison between the Effect of 1) Anti Angiotensin II 2) Angiotensin Inhibitor on the Prolonged Pressor Response to Renin in Nephrectomized Rats and 3) That Obtained by the Inhibitor on Angiotensin Induced High Blood Pressure Level

The results of all the therapeutic experiments described above are given in Fig 6A C in which each experiment is represented by a column The sum of the white and black part of each column indicates the blood pressure increase in mm Hg above the pre injection level of renin (A and B) or angiotensin (C) at the time at which treatment with anti angiotensin (A) or inhibitor (B and C) was started The hypotensive effect of the treatment is indicated by the height of the black column the top of the white column thus marking how many mm the blood pressure still exceeded the pre renin (or angiotensin) injection level at the maximum effect The interval between 1) the injection of renin or start of infusion of angiotensin and 2) the start of the treatment is plotted along the abscissa, differences in intervals are seen to be without any influence on the results obtained.

Although the hypotensive effect of the treatment is significant in all 3 groups, the comparison shows that the hypotensive effect of anti angiotensin II (A) is less than that of the angiotensin inhibitor (B) which in most cases results in a fall in the blood pressure to levels only about 5 10 mm above the pre renin injection level It is further seen that treatment with the inhibitor results in a total or subtotal return of the blood pressure to the pre angiotensin infusion level in all but one of the rats receiving infusion of angiotensin (C)

DISCUSSION

I Cause of the Prolonged Pressor Action of Renin in Nephrectomized Rats

The present experiments have shown that both the primary and the many hours prolonged pressor effect of renin in nephrectomized rats is partly or totally abolished by treatment whether with anti angiotensin or with an angiotensin inhibitor (Fig 3 and 4) This effect is found even when the treatment is started 1 3 hours after the renin injection at which time the injected renin has been found to be eliminated from the blood (*Schaechtelin et al 1964* and *Peters Haefeli 1971*) A comparison between the effect of the 2 types of treatment shows that the effect of anti angiotensin in most cases is less pronounced than that of the inhibitor (Fig 6A and B respectively) Similar inhibitor treatment of the elevated blood pressure in rats continuously intravenously *infused with angiotensin* resulted in a further pronounced decrease in the blood pressure which in most cases returned to normal values (Fig 6C) as previously observed by *Pals et al* (1971)

On the assumption that the above mentioned determination of the rate of disappearance of injected renin is correct the finding that *the prolonged renin pressor response of binephrectomized rats in any case is due mainly to a continued effect of renin* raises the question whether the prolonged effect may

be attributable to an accumulation of renin in the arterioles, as proposed by *Schaechtelin et al* (1964) The common finding of a more pronounced effect if the more easily diffusible inhibitor is used in the treatment rather than the much higher molecular antibodies (anti-angiotensin II), speaks in favour of this explanation

II More Prolonged Pressor Effect of Renin in Conscious than in Anaesthetized Normal Rats

It has previously been found that anaesthesia impairs the pressor effect of renin (see *Page & McCubbin* 1968, and *Lee* 1969), the *height* of the response being markedly smaller in the anaesthetized animals In the present study, however, the height was about the same in conscious and amytal anaesthetized rats (Fig 1), the latter is in agreement with findings in some recent experiments on dogs (*Assaykeen et al* 1968) and with the results obtained by *Goldblatt et al* (1972) after continuous renin infusion at low rates, on the other hand, their experiments showed that a single large dose gave a higher pressor response in conscious than in anaesthetized *dogs* The cause of the marked difference in *lengths* of pressor response in conscious and in anaesthetized rats is unknown The length of the response in conscious rats is close to what would be expected according to the rate at which injected renin disappears (the broken line in Fig 1) The practical consequence of this difference is that anaesthetized rats are better suited for renin-assay, since changes in sensitivity of the test animals will be of minor importance if intervals between injections are short

---

Supported by grants from *King Christian X's Foundation* The Tybjærg Hansen transducer and the Servogor recorder were given to the Institute by grants from the *Danish Foundation for the Advancement of Medical Science*, *Nordisk Insulinfond* and the *legacy of Miss P A Brandt*

REFERENCES

Assaykeen, T A, Otsuka, K & Ganong, W F Rate of disappearance of exogenuous dog renin from plasma of nephrectomized dogs. Proc Soc. Exp Biol Med *127* 306–310, 1968.

Basso, N & Taquini, A C Changes in the renin angiotensin system after bilateral nephrectomy Acta Physiol Latino-Amer *20* 22–27, 1970

Bing, J & Poulsen, K Effect of anti angiotensin II on blood pressure and sensitivity to angiotensin and renin Acta path microbiol scand A *74* 139–140, 1968 and *78* 6–18, 1970

Goldblatt, H, Haas, E & Haas, R Studies on renin Circ. Res. *31* 74–82, 1972

Houssay, B A, Braun Menendez, E & Dexter, L Destruction and elimination of renin in the dog Annals Int Med *17* 461–473, 1972

Lee, M R Renin and hypertension, London 1969, page 76

Page, I H & McCubbin, J.W Renal hypertension Chicago 1968, page 30, 126 and 255

Pals, D T, Masucci, F D, Denning, G S, Sipos, F & Fessler, D C Role of the pressor action of angiotensin II in experimental hypertension Circ Res *29* 673–681, 1971

Peters Hoefeli, L Rate of inactivation of endogenous or exogenous renin in normal and in renin depleted rats Am J Physiol *221* 1339–1345, 1971

Popovic, V & Popovic, P Permanent cannulation of aorta and vena cava in rats. J Appl Physiol *15* 727 728, 1960

Schaechtelin, G, Regoli, D & Gross, F Quantitative assay and disappearance rate of circulating renin Am J Physiol *206* 1361–1364, 1964

Schneider, E G, Rostorfer, H H & Nash F D Distribution volume and metabolic clearance rate of renin in anaesthetized nephrectomized dogs Amer J Physiol *215* 1115–1122 1968

Tigerstedt R & Bergmann P G Niere und Kreislauf Skand Arch Physiol *8* 223–238 1898

Acta path microbiol scand Section A 81, 3, 254–262, 1973

ROLE OF THE RENIN-SYSTEM IN NORMO- AND HYPERTENSION

Effect of Angiotensin-Inhibitor (1-Sar-8 Ala-Angiotensin II) on the Blood Pressure of Conscious or Anaesthetized Normal, Nephrectomized and Renal Hypertensive Rats

JENS BING and KNUD NIELSEN

The University Institute for Experimental Medicine, Nørre Allé 71, 2100 Copenhagen Ø

Infusion of the angiotensin II inhibitor 1 Sar 8-Ala angiotensin II causes a hypotensive effect in anaesthetized rats but not in conscious normal rats, it lowers the blood pressure both in anaesthetized and in conscious rats with renal hypertension due to clamping of one renal artery, no matter whether the opposite kidney remains intact or it has been removed. In the latter type of hypertension, higher doses are apparently needed These findings indicate that the renin angiotensin system plays a role both for the maintenance of the normal blood pressure and in the pathogenesis of both types of renal hypertension Infusion of the inhibitor results in a primary transient short pressor response, which in nephrectomized animals is smaller than that in normal rats, being even smaller or totally absent in renal hypertensive rats.

Despite the numerous studies on the part played by the renin system in the maintenance of the blood pressure level in normal and hypertensive animals as well as in humans, the question is still unsolved Determination of the plasma levels of renin and angiotensin and studies on the effect of blockade with antibodies and inhibitors are the main ways of elucidating the problems (see *Page & McCubbin* 1968, *Lee* 1969, *Wilson et al* 1971 *Gross* 1972)

The aim of the present paper is to elucidate the problems by means of a synthetic peptide (Sar Arg Val-Tyr-Val His Pro-Ala), it is a specific competitive inhibitor of angiotensin II, the effect of which has previously been studied by *Pals et al* (1971) and *Brunner et al* (1971) The present paper is a report on the effect of this inhibitor which was studied on conscious or anaesthetized normal or nephrectomized rats and on 2 types of renal hypertensive rats, in some cases, the hypertension was due to clamping of one renal artery while the opposite kidney remained untouched, ("two kidney hypertension"), in other cases it was due to clamping of one renal artery after removal of the other kidney ("one kidney hypertension") The results of studies on the effect of the inhibitor during continuous infusion of angiotensin and on its effect on the prolonged pressor effect of renin in bi-nephrectomized rats have been reported in a separate paper (1973)

Received 1 xii 72 Accepted 1 xii 72

MATERIAL AND METHODS

The experiments were performed on female Wistar S P F rats of about 200 g using the angiotensin inhibitor 1 Sar 8 Ala angiotensin II from the Norwich Pharm Comp New York The method

used for blood pressure determination is mentioned in detail in the preceding paper (*Bing* and *Nielsen* 1973) Renal hypertension was induced by partial clamping of one renal artery, using the method of *Wilson* and *Byrom* (1939) in some animals the procedure was followed by removal of the opposite kidney Intraperitoneal injection of 100 mg/kg b w amobarbital was used for prolonged anaesthesia while ether was used for the short term anaesthesia required for the performance of the operations Some of the rats were pretreated with 0 005 mg ergotamine tartrate

RESULTS

I *Effect of the Angiotensin Inhibitor on Conscious and Anaesthetized NORMAL Rats*

In all of 9 experiments on 6 *conscious normal* rats, 20 30 minutes infusion of 10 μg/kg b w /min inhibitor, in one case increased up to 20 μg during the infusion, resulted in a *pressor response,* the blood pressure rose quickly by about 30-40 mm Hg above the pre infusion level upon which it returned more slowly to this level, either during the infusion or shortly after it was discontinued (Fig 1A) Discontinuation of the infusion did not result in any increase in the blood pressure

The effect of 15 30 minutes infusion of 10, or in 2 rats of 50 μg/kg/min inhibitor in 22 experiments on 16 *anaesthetized normal* rats gave in most cases (11 rats) a characteristic response (Fig 1B) starting with a transient *primary pressor response of about 10 20 mm* which after a few minutes was followed by a *secondary depressor effect to 10-15 mm below the pre infusion level* This low level was maintained during the rest of the infusion period *After termination of the infusion the blood pressure rapidly increased not only to the pre infusion level but most often up to 5 12 mm in one case up to 20 mm above the pre infusion level* (Fig 1B) The 2 rats which were infused with the higher dose (50 μg) responded in the same way but the pressor response was a little higher (23 and 30 mm Hg) while the secondary depressor effect was the same as that found with the smaller dose

By way of comparison with the hypotensive effect caused by the inhibitor, examples of the similarly deep, but shorter depressor effect of anti angiotensin II in anaesthetized normal rats are marked with broken lines in Fig 1B, these curves deriving from previous studies (*Bing & Poulsen* 1968 and 1970) A minority consisting of 5 rats, 3 of which were tested in the afternoon, showed the primary pressor effect, but not the secondary depressor-effect. The type of response did not depend on whether or not the rats had been pretreated with ergotamine tartrate

Both in conscious and in anaesthetized rats, the *sensitivity to angiotensin* decreased to a-

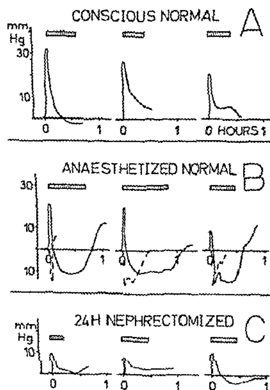


Fig 1 Effect of infusion of 10 μg/kg/min (▭) of the angiotensin II inhibitor 1 Sar 8 Ala-angiotensin II on the blood pressure of conscious (A) and amytal anaesthetized (B) normal rats and of 24 hour nephrectomized (C) rats in which the results obtained in normal and anaesthetized rats were identical In Fig 1B the result of treatment of anaesthetized normal rats with anti-angiotensin II is indicated by broken lines the curves deriving from a previous study (1968 and 1970) The ordinate shows the changes in blood pressure in mm Hg the time in hours being plotted along the abscissa.

bout 1 per cent of that found before the infusion of inhibitor, both during the infusion and even after its discontinuation, which is in contrast to the *rapid post infusion increase* in blood pressure to or above the starting level In 2 cases, a 25-50 per cent decreased sensitivity to angiotensin was still found more than one hour after infusion was discontinued

By way of comparison, 3 rats were infused with a 0 9 per cent sodium chloride solution instead of the inhibitor solution In these cases there was no change in blood pressure, neither during infusion nor after infusion was discontinued

II Effect of the Angiotensin-Inhibitor on Conscious or Anaesthetized, 24-Hour-NEPHRECTOMIZED Rats

In all of 7 experiments on six 24 hour-nephrectomized rats, infusion of 10 μg/kg/min inhibitor for 15-30 minutes resulted in a *primary, about 10 mm* (range 7-18 mm) *pressor response* of 2-5 min duration *upon which the blood pressure returned to, or close to, the pre infusion level* both during the remaining infusion and after the infusion was discontinued (Fig 1C) This type of reaction was found to be uninfluenced by anaesthesia

The *sensitivity to angiotensin* was first tested after the discontinuance of the infusion where it had decreased to levels seen in normal rats, values below 30 per cent of the primary sensitivity were found after about 10 *minutes and below 50 per cent after 30-50* minutes

III Effect of the Angiotensin-Inhibitor on Conscious or Anaesthetized RENAL HYPERTENSIVE Rats

A „Two kidney hypertension" The effect of infusion of the inhibitor on the high blood pressure was studied in 18 experiments on 7 rats with renal hypertension, either 1 month (2 rats) or 4 months (5 rats) after clipping of one renal artery while the opposite kidney was left intact The animals were conscious in about half of the experiments in the other

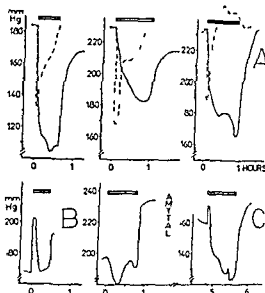


Fig 2 Effect of infusion of 10 ( ), 20 ( ) or 50 ( ) μg/kg/min of the angiotensin inhibitor on rats with *two kidney hypertension*, caused by clamping of one renal artery, the opposite kidney remaining intact Fig 2A gives examples of the marked hypotensive effect (without primary pressor response) in 3 rats Fig 2B shows that there is a primary pronounced pressor effect in a fourth rat, but no hypotensive effect which in this case may have been masked by a simultaneous, spontaneous rise in blood pressure the latter probably being responsible for the marked post infusion increase in blood pressure above the pre-infusion level A still more marked and rapid post infusion increase in blood pressure is seen in the first curve in figure 2C showing the effect of the infusion of inhibitor on a rat which was studied *first in the conscious state and later on the same* day under amytal anaesthesia which resulted in a decrease in blood pressure In the experiments on this rat the dose was increased from 10 20 μg/kg/min the result being a minor increase in the hypotensive effect The blood pressure in mm Hg, is plotted along the ordinate while the time in hours is plotted along the abcissa The *result of* treatment with anti angiotensin II is indicated by broken lines

half they were anaesthetized In some cases the same rats were tested on two occasions, first in the conscious state and about 3 hours later, in amytal anaesthesia Neither pretreatment with ergotamine tartrate nor anaesthesia brought about any significant change in the response In 3 rats which were studied on

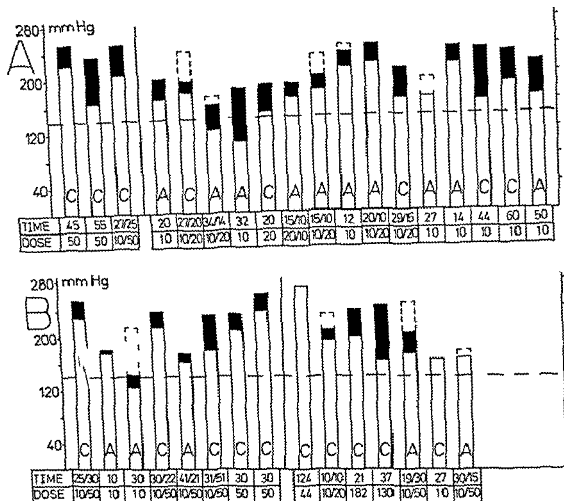


Fig. 3. The result of the hypotensive effect of the angiotensin II inhibitor in all experiments on "two kidney" (3A) and "one kidney hypertension" (3B) is given by marking the individual experiments with a column. The sum of the white and black part of each column indicates the blood pressure level in mm Hg *before the infusion, the height of the black column* indicating the maximum hypotensive effect of the infusion, thus the top edge of the white column shows the lowest blood pressure obtained during infusion. The columns drawn with broken lines on top of the black column indicate the level above the preinfusion level to which the blood pressure rose in some cases when the infusion was stopped as shown in Fig. 2B and C. *The doses infused* in µg/kg/min and the time of the infusion are given below each column. If changing doses were given both values separated by a diagonal line are given. The vertical lines indicate differences in time after application of the clamp on the renal artery, the interval covering 1 and 4 months in two-kidney hypertensive rats and 11 15 days and 1 2 months respectively in the one kidney hypertensive rats. The horizontal broken line indicates the upper limit of the normal blood pressure. The letters on the white columns indicate whether the rats were anaesthetized (A) or conscious (C).

2 4 successive days, depression of the blood pressure was most intense on the first day. The dose of inhibitor was in most cases 10 µg/kg/min. In 5 experiments the dose was increased to 20 µg, which in only one case caused a further fall. In 2 rats infused with 50 µg/kg/min the response was not significantly higher than that in rats which received only 10 µg, but in a second experiment on one of the latter the blood pressure fell by 32 mm (from 252 220

mm) during a 27-minute infusion of 10 μg and a further fall by 13 mm (to 207 mm) was observed after the dose had been increased to 50 μg/kg/min The duration of the infusion was in most cases 25-50 minutes

The effect of the angiotensin inhibitor on "two kidney hypertension" is seen in Fig 2A which gives examples of its marked hypotensive effect on these hypertensive rats and by way of comparison, examples (broken lines) of the almost equally deep, but shorter hypotensive effect of anti-angiotensin, as previously observed (1970). Among the total number of cases, a few failed apparently to present any hypotensive effect of the infusions (Fig 2B), or such effect was only slight (first treatment in Fig 2C), but in these cases, discontinuance of the infusion resulted in a rise in blood pressure to values markedly above that found at its start The lack of (B), or the small (C), hypotensive effect in such cases seems, therefore, to be due to summation of 1) a depression caused by the infusion of inhibitor and 2) a spontaneous increase in blood pressure which more or less balance each other The degree of the hypertension at the start of the treatment, the maximum depression of the blood pressure during the infusion of the inhibitor, and the occasional increase after discontinuance of the infusion to values higher than those found before its start are given for all the experiments in Fig 3A In this figure the sum of the white and black columns indicates the blood pressure level before the infusion, the top edge of the white columns gives the pressure at the maximum effect of the treatment, and the top edge of the columns, in some cases drawn with broken lines, the levels to which the blood pressure rose after discontinuance of the infusion, as *shown in the examples seen in Fig 2B and C* In Fig 3A it is further indicated whether the rats were conscious (C) or amytal anaesthetized (A) at the time of the experiment, the dosage of inhibitor in μg/kg b w /min and the duration of the infusion in minutes are given below the columns The maximum effect of the treatment was (independent of the dosage) achieved at different intervals of time covering from 6 to 55 minutes after the start of the infusion Although the inhibitor is seen to cause a depressor effect in all experiments, the blood pressure

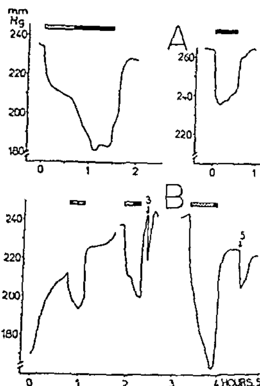


Fig 4 Effect of infusion of the inhibitor on rats with *"one kidney hypertension"* caused by clamping of one renal artery and removal of the opposite kidney Fig 4A gives examples of the marked hypotensive effect (without primary pressor response) in 2 rats, the first curve further showing a marked effect if doses were increased from 10 (▭) to 50 (▬) μg/kg/min Fig 4B shows the blood pressure of a third rat which before treatment had a spontaneous increase in blood pressure, which continued during the following hours. This rat, which was conscious throughout the experiment received 3 infusions the doses being 10 (▭) to 20 (▭) in the first, 20 to 50 (▬) in the second (on each occasion showing increasing effect with increasing dose) and 100 to 200 (VVVV) in the third infusion which terminated by infusion of 50 μg/kg/min during the last 5 minutes, the decline in dose resulted in a small increase in blood pressure In addition to the 3 infusions 2 quick injections of 3 and 15 μg of the inhibitor resulted in marked hypotensive responses The ordinate shows the blood pressure in mm Hg while the time in hours is plotted along the abscissa

was lowered to normal values only in 2 cases (below 140 mm), in another 2 experiments it ranged between 140 and 160 mm, in 14 above 160, and in 5 of the latter above 200 mm at the maximum effect of the treatment. In only 4 out of the 18 experiments, a 7-16 mm primary pressor effect of about 2 minutes' duration was found at the start of the infusion.

B "One kidney hypertension". The effect of the angiotensin inhibitor was further studied in 15 experiments on 10 rats with renal hypertension either 11-15 days or 2-2½ months after unilateral nephrectomy and clipping of the opposite renal artery. As in the studies on "two kidney hypertension" there was no significant difference between the results obtained in conscious and anaesthetized rats. The doses given to these rats were increased from 10 to 50 μg/kg/min, in 2 cases even more, the duration of the infusion being 20-50 minutes in most cases. In several cases, the dosage was changed during the infusion.

The fact that even rats with "one kidney hypertension" (with 2 exceptions) reacted with a hypotensive response to infusion of the angiotensin inhibitor is seen in Fig. 4A-B and Fig. 3B. Fig. 4A shows the most common type of response when doses of 50 μg/kg/min were used together with the further increase in response when the dose was changed from 10 to 50 μg. The response in a third rat in which the catheters had been placed in the artery and vein on the preceding day, and in which a small bleeding from the wound occurred during the night, is given in Fig. 4B. In this case, the pressure was relatively low in the morning, probably because of the bleeding, but it rose spontaneously during the day to values above 240 mm Hg. During the experiment lasting for about 5 hours, the rat received 3 infusions of the inhibitor, on each occasion by changing doses, ranging from 10 to 20 μg on the first occasion and from 20 to 50 μg on the second resulting in an augmentation of the response, as in the experiment shown in Fig. 4A. On the third occasion much higher doses of 100-260 μg were given, resulting in a correspondingly much deeper, depressor effect. In addition to the 3 infusions, quick injections, first of 3 μg and later of 15 μg of the inhibitor were given, also resulting here in marked hypotensive effect.

A survey of all results obtained when one kidney hypertensive rats were treated with infusions of the angiotensin inhibitor is given in Fig. 3B, the symbols being the same as those used in the survey of the results on two kidney hypertension in Fig. 3A.

A comparison of these 2 figures shows a significant decrease in blood pressure in both types of renal hypertension, but the response seems to be somewhat less marked in "one kidney" hypertension, than in "two kidney" hypertension, although the rats with "one kidney hypertension" most often received higher doses of the inhibitor. Only 2 rats failed to respond, one of these received only 10 μg/kg/min, while the other, which received a very high dose, was the only rat in this group, which was *not* pretreated with ergotamine tartrate. The figure shows finally that it was only in one experiment that treatment resulted in a normal blood pressure, the pressure being above 160 mm in all the others, and even above 200 mm in 4 of these at the maximum effect of the treatment. A primary pressor effect of about 6-20 mm during the first approximate 5 minutes of the infusion of the inhibitor was seen in 4 out of the 15 experiments.

DISCUSSION

I. Role of the Renin-System in Normotension

Because of the extremely complicated regulation of the circulation (see Guyton et al. 1972) it has been difficult to obtain information about the role of the renin-system for maintenance and physiological variations in the blood pressure in normotensive animals. Evidence that renin participates in the maintenance of the blood pressure has been given in recent studies showing that *anti angiotensin* evokes a hypotensive response in anaesthetized normal rats (*Bing & Poulsen* 1968, 1970, *Worcel et al.* 1969, and *Brunner et al.* 1971, 1972). It has further been shown that

even low rates of renin infusion *result in* blood pressure elevations which are related to the changes in plasma renin concentrations (*Bianchi et al* 1968, and *Goldblatt et al* 1972) It has also been shown that the kidneys respond to hypotension by a rapid release of renin in amounts sufficient to contribute to the short term blood pressure regulation (*Opard et al* 1970, and *Cowley et al* 1971 and 1972) Further evidence for the participation of renin in the maintenance of the normal blood pressure emerges from the fact that a small depression in the blood pressure (4 mm) was found in the angiotensin inhibitor treated pithed rat (*Pals et al* 1971) and from the finding in the present study that the hypotensive effect of the angiotensin inhibitor was pronounced in most of the *anaesthetized* normal rats but not in the binephrectomized rats (Fig 1) The lacking hypotensive effect of the inhibitor in *conscious* rats (Fig 1) is in aggreement with previous findings in conscious rats whether treated with angiotensin inhibitors (*Pals et al* 1971) or with anti angiotensin it may be ascribed to an interaction of homeostatic pressure regulation systems which are more or less inactivated by anaesthesia

II Role of the Renin System in Renal Hypertension

One of the many methods by which the pathogenesis of renal hypertension may be elucidated includes blockage of the different factors involved in the renin system by which it may be shown whether such blockage will result in a normalization of the blood pressure Many types of blocking of the renin angiotensin system have been used for this purpose including subtotal depletion of renal renin and treatment with antirenin anti angiotensin II inhibitors of either renin converting enzyme or angiotensin II and with angiotensinase (For references cf the reviews by *Page* & *McCubbin* 1968 *Gross* 1972 and *MacDonald et al* 1972) The very different results obtained are probably partly due to the incomplete blockage obtained by some of the methods used and partly to the fact that both the test animals as well as types of hypertension were different These causes are evidently not the only reason why different results have been obtained as different results have also been obtained in several cases in which the same method of blockage had been used on the same type of hypertension in the same species of experimental animal

Such differences have also been found when the blockage as in the present study was performed with the angiotensin II inhibitor 1 Sar 8 Ala angiotensin a specific competitive antagonist to the vascular action of angiotensin II Using 1 hour infusion of 10 μg/kg/min of this angiotensin inhibitor in endocrine kidney hypertensive conscious rats *Pals et al* (1971) found that the blood pressure was depressed to normal values only during the acute phase (about 7 days) while there was no significant effect during the chronic phase (4 5 weeks) Contrary to this, *Brunner et al* (1971) who infused the same dose during the same period of time in the chronic phase of hypertension (6 weeks) found a marked effect on anaesthetized two kidney Goldblatt hypertensive rats The blood pressure fell from about 200 to about 160 mm while the changes in blood pressure induced in the one kidney type of renal hypertension were so small that it was thought to be insignificant They concluded on the basis of these findings that 2 different mechanisms might be in evidence a causal role for the renin system being found in the two kidney hypertension but not in the one kidney hypertension A similar conclusion was drawn by *Brunner et al* (1972) who used an anti angiotensin and by *Krieger et al* (1971 1972) who used a pentapeptide known to block the *in vivo* conversion of angiotensin I to angiotensin II So far the results obtained in the present study differ from previous findings in that they show that *the angiotensin II inhibitor induces a depression of the blood pressure in both acute and chronic renal hypertension of the two kidney Goldblatt type as well as the one kidney Goldblatt type* (Fig 2 4) Although this result in several cases was

obtained with the same doses as those used by the 2 former authors, the fact that higher doses were used in many of our other experiments may be the reason why a more pronounced depressor response was achieved, especially in "one kidney hypertension" rats Thus, the degree of the fall in blood pressure was repeatedly seen to increase with increasing dose (Fig 4A and B) and the most pronounced effect was obtained with the largest dose (Fig 4B) No doubt, still higher doses applied during longer times of infusion may result in a further normalization of the blood pressure Although the inhibitor brings about a hypotensive effect in both types of renal hypertension, the two types seem to differ, as shown also in the study of *Brunner et al* (1971), as higher doses are often required in the 'one kidney' type, and even with a large dose one rat failed to respond The hypotensive effect found in cases in which such effect is small cannot be regarded as insignificant, since comparison with findings in all conscious normal rats shows a total absence of hypotensive effect

The finding that the renin-system plays a role not only in the 'two kidney type" of renal hypertension in which plasma renin often is increased, but also in the one kidney type" in which normal plasma renin is found (*Gross et al* (1965), *Koletsky et al* (1971)) supports the hypothesis that normal plasma renin level may be inappropriate to total body sodium and blood pressure in some cases of hypertension (*Lee* 1969) The normal plasma renin levels found in animals with one kidney hypertension are not found in the initial phase where renin levels have been found to be increased during the first hours after constriction of the renal artery in rats (*Koletsky et al* 1971) as well as in dogs (*Miller et al* 1972) Miller et al (1972) found that blockage of the converting enzyme at this stage would restore the systemic pressure to normal

If the result of the angiotensin inhibitor infusion be compared with that obtained after injection of 50-300 μl serum containing angiotensin II antibody during approximately 10 seconds, the rate of depressor-effect of the antibody will be seen to be more abrupt, but of much shorter duration in normal (Fig 1B) as well as in hypertensive (Fig 2A) rats The more rapid decrease is a natural consequence of the more rapid injection, but it is difficult to explain why it is of shorter duration, as the pressor action of exogenous angiotensin II remains blocked for a long time both in anti angiotensin and angiotensin inhibitor treated rats

III Primary Pressor Effect of the Angiotensin Inhibitor

Pals et al (1971) found that infusions of 1 Sar 8-Ala angiotensin II in doses of 1 μg/kg/min caused an initial small (3-4 mm Hg) and transient (4 5 minutes) elevation of the baseline blood pressure in pithed rats The present study shows that higher doses (10 50 μg/kg/min) result in higher elevations which are dose dependent These primary increases in blood pressure are some what higher in conscious than in amytal-anaesthetized rats and, in accordance with previous studies on the sensitivity to angiotensin II (*Bing* 1969), the pressor effect of the inhibitor was found to be smaller in nephrectomized rats (Fig 1) and still smaller, or totally absent, in renal hypertensive rats

---

Supported by grants from *King Christian X s Foundation* The Tybjærg Hansen transducer and the Servogor recorder were given to the Institute by grants from the *Danish Foundation for the Advancement of Medical Science Nordisk Insulinfond* and the *Legacy of Miss P A Brandt*

REFERENCES

Bianchi, G, Brown, J J, Lever, A F, Robertson, J I S & Roth, N Changes of plasma renin concentration during pressor infusions of renin Clin Sci 34 303–314 1968

Bing J Changes in sensitivity to angiotensin and renin Acta path microbiol scand 77 609–616 1969

Bing, J & Poulsen, K Effect of anti angiotensin on blood pressure and sensitivity to angiotensin and renin Acta path microbiol scand A 74 139–140, 1968, and 78 6–18, 1970

Brunner, H R, Kirshman, J D, Sealey, J E & Laragh, J H Hypertension of renal origin Evidence for two different mechanisms. Science *174* 1344–1346, 1971

Brunner, H R, Chang, P, Wallach, R, Sealey, J E & Laragh, J H Angiotensin vascular receptors. J Clin Invest *51* 58–67, 1972

Cowley, A W, Miller, J P & Guyton, A C Quantification of intermediate steps in the renin angiotensin vasoconstrictor feed back loop Circ Res. *28* 568–581, 1971, and *30* 557–566, 1972

Goldblatt, H, Haas, E & Haas, R Studies on renin Circ Res. *31* 74–82, 1972

Gross, F, Brunner, H & Ziegler, M Renin angiotensin system, aldosterone and sodium balance Rec Progr Hormone Res. *21* 119–177, 1965

Gross, F Niere und Hochdruck Klin Wschr *50* 621–635, 1972

Guyton, A C, Coleman, T G & Granger, H J Circulation, overall regulation Ann Rev Physiol *34* 13–46 1972

Koletsky, S, Pavlicko, K M & Rivera Velez, J M Renin angiotensin activity in hypertensive rats with a single ischemic kidney Lab Invest *24* 41–44, 1971

Krieger, E M Salgado, H G, Assan, C J, Greene L L & Ferreira, J H Potential screening test for overactivity of renin angiotensin system. Lancet 1 269–271, 1971, and Circ. Res. *31* Suppl II 62–71, 1972

Lee, M R Renin and hypertension London 1969 page 139–170

McDonald, G J, Boyd, G W & Peart, W S Renal hypertension and angiotensin antibodies Am Heart J *83* 137–139, 1972

Miller, E D, Samuels A J, Haber, E & Barger, A C Inhibition of angiotensin conversion in experimental renovascular hypertension. Science *177* 1108–1109, 1972

Oparil, S, Vassaux, C, Sanders, C A & Haber, E Role of the renin in acute postural homeostasis Circulation *41* 89–95, 1970

Page, I H & McCubbin, J W Renal hypertension Chicago 1968, page 230–232

Pals, D T, Masucci, F D, Denning G S, Sipos, F & Fessler, D C Role of the pressor action of angiotensin II in experimental hypertension. Circ Res *29* 664–681, 1971

Wilson, C, Ledingham, J M & Floyer, M A Experimental renal and renoprival hypertension In *Rouiller, C & Muller, A F* The kidney *IV* 155–247, 1971

Worcel, M, Meyer, P, d Auriac, D A & Milliez, P Role of angiotensin in normal blood pressure regulation Pflugers Arch *310* 251 263, 1969

Acta path microbiol scand Section A 81, 3, 263 268, 1973

RENIN IN DIFFERENT TISSUES, AMNIOTIC FLUID AND PLASMA OF PREGNANT AND NON-PREGNANT RABBITS

PETER CLAES ESKILDSEN

The University Institute for Experimental Medicine
(Head Jens Bing M.D), Copenhagen, Denmark

In rabbits pregnancy causes a marked increase in the renin concentration of plasma and several organs uterus, vagina, mesometrium, liver, ureter, heart, arteries and veins, muscle and fat tissue while the renin content of the kidney, oviduct ovary and adrenal gland is the same in pregnant and non pregnant rabbits. There is evidence for a local renin formation in kidney, uterus oviduct and in the chorion and amnion The occurrence of renin in the other tissues may either be a result of local formation or due to uptake of circulating renin The rabbit brain is not found to contain measurable amounts of renin

Different organs besides the kidneys are known to contain renin (for literature see *Page & McCubbin* 1968, *Lee* 1969) In the rabbit special studies of the renin content of the pregnant female genital tract have been performed (*Gross et al* 1964, *Bing & Faarup* 1966, *Eskildsen 1972*[a])

On the basis of these reports and a previous observation (*Eskildsen* 1973) of not negligible concentrations of renin in the *ureter*, abdominal wall and omentum majus of pregnant, but not of non pregnant, rabbits, it is attempted in the present investigation to study whether the renin concentration is significantly elevated above the plasma renin concentration in a number of organs (and in amniotic fluid) from non pregnant and pregnant rabbits Also the arterial and uterine venous plasma renin are estimated

Received 21.xii 72 Accepted 21 i 73

Requests for reprints should be addressed to *Peter Claes Eskildsen*, The University Institute for Experimental Medicine Nørre Allé 71 2100 Copenhagen Ø Denmark

MATERIALS AND METHODS

Animals Five non pregnant and 7 28 days pregnant, albino country rabbits (the Statens Seruminstitut), weighing 3 5-4 5 kg were used The findings in 8 non pregnant rabbits given in a previous paper (*Eskildsen* 1972[b]), are included in the study

The *tissue* was sampled immediately after the animals were killed either during anaesthesia (pentobarbital sodium 40 mg/kg) and bleeding or by intravenous injection of air Tissue was taken from the following parts of the *genital tract* uterine wall (the whole circumference of an uterine segment between two placental sites)—oviduct (one oviduct in toto)—vagina (biopsy)—mesometrium (biopsy)—ovary (one)—foetal and maternal placentae (biopsies)—foetal membranes chorion and amnion (biopsies)—amniotic fluid (pooled from several sacs in each case) Biopsies were further taken from the following other *organs* kidney (cortex + medulla)—adrenal gland (one gland)—liver—brain hemisphere and brain stem—heart (left ventricle)—ureter *vessels* abdominal aorta—inferior vena cava—renal artery—renal vein—

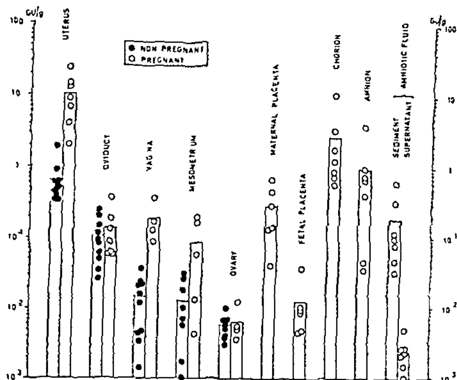


Fig 1. The renin concentration, GU/g in logarithmic scale, in the uterus, oviduct, vagina, mesometrium, and ovary, maternal placenta, foetal placenta, chorion, amnion, sedimental and supernatant fractions of the amniotic fluid of 10 non-pregnant and 7 28 days pregnant rabbits The individual values as well as the means (columns) are shown

mesenteric vessels (arteries and veins from which fatty tissue was coarsely released), and *muscle and fatty tissue* abdominal wall—diaphragma—omentum majus—subcutaneous fat

The *tissue samples,* stored at 20°C, were exposed to homogenization, extraction and transitory acidification prior to the renin determination as previously described (*Eskildsen* 1970)

The *amniotic fluid* was centrifuged at 1000 g immediately after collection The thin layer of sediment was resuspended in 100 μl saline before storage at 20°C Both components were treated as the tissue samples before the renin determination

The *blood* (1–2 ml) was withdrawn from the ear artery of 18 non-pregnant and 16 terminal pregnant conscious rabbits During anaesthesia *uterine venous* blood was obtained from 8 pregnant animals through a catheter in a mesometrial vein stem Peripheral blood (from ear or carotic artery) was in these cases taken shortly before and a few minutes after the sampling of uterine venous blood

The *plasma samples,* stored at –20°C as citrate plasma (50 μl 6 per cent sodium citrate per 1 ml blood), were transitory acidified in order to inhibit the angiotensinase activity according to a modification (*Poulsen* 1971) of the method used for the tissue extracts

The *renin assay* was based upon the principle of determination of the decrease in angiotensinogen concentration in the course of time (*Poulsen* 1968), using radioimmuno assay for the estimation of angiotensin I (*Poulsen* 1969, *Eskildsen* 1972)

The *renin concentration* is expressed in *Goldblatt Units* per gram (Gu/g) by reference to a highly purified hog renin preparation (WHO, Laboratory for Biological Standards Mill Hill London) The plasma renin concentration is determined as previously described (*Eskildsen* 1973) using the integrated form of the Michaelis equation and, after estimation of the rate constant for the enzymatical reaction between renin and renin substrate (*Poulsen* 1971), expressed in Gu $\times$ 10 $^{-4}$/ml In spite of the difference in substrate concentration identical ($p > 0.50$) values of the rate constant k' were measured in plasma-pools from non pregnant (k' = 131 6 ml/GU $\times$ hr (S E 8 8 ml/Gu $\times$ hr n = 5)) and pregnant animals (k' = 125 5 ml/GU $\times$ hr (S E 3 8 ml/GU $\times$ hr, n = 6)) The plasma renin substrate concentration is indicated as nanograms angiotensin I per ml plasma.

The *histological examination* of film preparations of the sediment of amniotic fluid was performed after staining with May Grunwald Giemsa and PAS (*Petri* 1968) stains

RESULTS

1 *Renin in Different Tissues from the Genital Tract of Pregnant and Non Pregnant Rabbits*

Considerable concentrations of renin were present in the *uterus* of the 7 terminal pregnant rabbits (Fig 1), on an average they were 16 fold higher than those of 10 non-pregnant animals, exceeding by 6 fold the renal renin concentration (Fig 2) The renin concentration in the *oviduct* was unchanges by pregnancy, while the low renin concentrations, measured in the *vagina* and the *meso metrium* of non pregnant animals, were increased several times in the pregnant animals The *ovaries* of pregnant and non-pregnant animals contained equally small amounts of renin

Rather high renin concentrations were found in the *foetal membranes*, especially in the *chorion*, which on an average showed 3 fold higher values than the *amnion*, but 3–4 fold lower than the uterus The renin concentration in the *foetal placenta* was much lower and exceeded by several fold that in the *maternal placenta*

In the *amniotic fluid*, the renin concentration in the *sediment* was much higher than that in the *supernatant*

The *histological examination* of film preparations of the amniotic fluid-sediment showed, besides a few erythrocytes, large, pale, epithelial, polygonal cells The nucleus was large and oval with nucleoli, while the cytoplasma was almost structureless and devoid of light microscopically visible PAS-positive granules

2 *Renin in Different Tissues Outside the Genital Tract in Pregnant and Non Pregnant Rabbits*

Estimation of renin concentration was performed on a number of organs and tissues removed from 5 non-pregnant and 7 terminal pregnant rabbits (Fig 2) The *renal* renin concentration, showing by far the highest values of all the studied samples, was about the same in pregnant and in non-pregnant

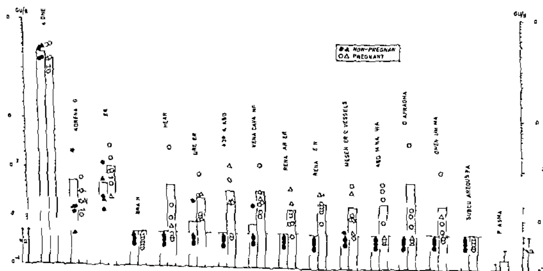


Fig 2 The renin concentration, GU/g in logarithmic scale, in different organs vessels, and tissues from 5 non pregnant (black symbols) and 7 terminal pregnant rabbits (white symbols) The animals were killed before the tissue sampling, either during anaesthesia (indicated by circles) or in the conscious state (indicated by triangles) Values below 5 × 10⁻⁴ GU/g were not further determined but placed in the "not detectable (N D) area" below the dotted line During calculation of the means (columns), these unmeasurable values were put at 5×10^{-4} GU/g The renin concentration, GU × 10^{-4}/ml, in peripheral arterial plasma from 18 non pregnant and 16 pregnant rabbits is shown to the right

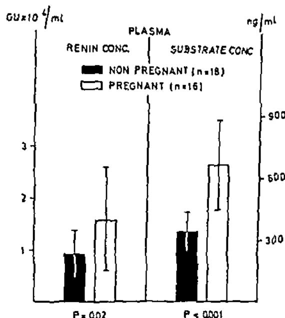


Fig 3 The mean (± S D) plasma renin concentration GU × 10⁻⁴/ml, and the mean (± S D) plasma renin substrate concentration ng angt/ml, determined in 18 non pregnant (black columns) and 16 28 days pregnant rabbits (white columns)

animals Much lower, but measurable values *of renin* were determined in the *adrenals* of non-pregnant and pregnant rabbits Similar renin concentrations were determined in the *liver* of non pregnant animals, while higher values were found during pregnancy No measurable amounts of renin ($<5 \times 10^{-4}$GU/g) were detected in the *brain*, either in the hemispheres or in the brain stem

The renin concentrations in the rest of the studied *organs, vessels* and *tissues* were unmeasurable in the *non pregnant* state but clearly increased during *pregnancy* showing values which were significantly elevated above the plasma renin concentration in terminal pregnant rabbits (Fig 2, to the right) Exceptions were few only In a single (anaesthetized) non pregnant animal increased renin concentration was found in the ureter and in the inferior vena cava Conversely, unmeasurable values of renin were found in the abdominal wall, diaphragma and omentum of some of the pregnant rabbits, especially in those killed by air embolism (Fig 2, indicated by triangles) The *subcutaneous fat* was found to be devoid of measurable amounts of renin both in pregnant and non pregnant rabbits

3 *Renin and Renin Substrate Concentration of Peripheral Arterial and Uterine Venous Plasma from Pregnant Rabbits*

The peripheral arterial *plasma renin concentration* was measured in 18 non pregnant and 16 terminal pregnant rabbits (Fig 3) Although the values showed great dispersion

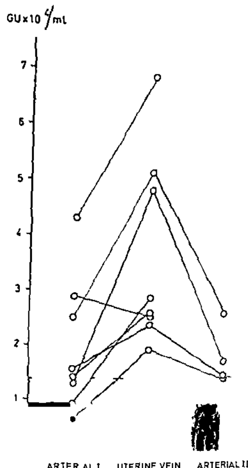


Fig 4 The individual plasma renin concentration GU × 10⁻⁴/ml in peripheral arterial plasma and uterine venous plasma from 8 terminal pregnant rabbits The arterial blood was sampled before (ARTERIAL I) and in 4 animals also a few minutes after (ARTERIAL II) the uterine venous blood was sampled The mean plasma renin concentration in non pregnant rabbits (0 92 GU × 10⁻⁴/ml) is indicated by the black area while the dotted line represents the upper limit of the standard deviation (± 0 46 GU × 10⁻⁴/ml)

a significant (Student's t test $p = 0.02$) increase in renin concentration was found in the pregnant animals (1.58 ± 1.02 (S D) GU $\times 10^{4}$/ml) as compared with the non pregnant animals (0.92 ± 0.46 (S D) GU $\times 10^{4}$/ml) The *plasma renin substrate concentration* in

± 96 (S D) ng angt /ml)

The *uterine venous plasma renin concentration* in 7 out of 8 terminal pregnant rabbits (Fig 4) was clearly elevated above the renin concentration of the *arterial* plasma, which showed almost identical values before and after (4 animals) the blood sampling from the uterine vein The arterial plasma renin concentration was in these anaesthetized animals the same as that found in conscious pregnant rabbits (Fig 3), and mainly above the plasma renin concentration in non-pregnant animals (Fig 4, black area)

DISCUSSION

In agreement with previous investigations (*Gross et al* 1964, *Bing & Faarup* 1966) the present study shows that high renin concentrations are demonstrable both in the maternal and the foetal tissue of the genital tract of pregnant rabbits. Besides the tremendous increase in uterine renin, a significant increase of the renin concentration in the vagina and mesometrium is observed, while the renin formation in the oviduct seems unaffected by pregnancy Although a transfer of renin from the rabbit uterus to intra uterine embedded autologous tissue has been demonstrated (*Eskildsen* 1972[b]), the high renin concentration in the chorion and amnion speaks for a local renin formation (*Symonds et al* 1968) This is further supported by the finding of the majority of the renin of the amniotic fluid in the cellular component which is shown to contain cells looking like exfoliated, epithelial amniotic cells (*Falck Larsen* 1964)

Pregnancy is furthermore shown to induce an increased occurrence of renin in a number of tissues outside the female genital tract in rabbits In agreement with previous findings (*Bing & Faarup* 1966), the renal renin remains unchanged during pregnancy, the same applies to the modest content of renin in the adrenal glands (*Ryan* 1967) The rabbit brain is, in contrast to the dog brain (*Fisher-Ferraro et al* 1971, *Ganten et al* 1971), devoid of measurable amounts of renin The subcutaneous fatty tissue is similarly without measurable concentration of renin both in pregnant and non pregnant animals The increase of hepatic renin during pregnancy is possibly caused by the increased level of plasma renin, as the liver is suggested to play a role in the elimination of renin (*Christlieb et al* 1968 *Barnardo et al* 1968, *Horky et al* 1970)

In the heart, ureter, vessels, muscle and fatty tissue of pregnant rabbits, measurable concentrations of renin, significantly elevated above plasma renin, are observed It remains a question whether this widespread occurrence of renin during pregnancy is caused by an increased uptake of circulating renin, or by a local renin formation

---

This work was supported by grants from *Nordisk Insulinfond* and the *Legacy of Miss P A Brandt*

REFERENCES

1 *Barnardo, D E, Strong C G & Baldus, W P* Failure of the cirrhotic liver of inactivate renin Evidence for a splanchnic source of renin like activity J Lab Clin Med *74* 495–506 1969

2 *Bing J & Faarup, P* A qualitative and quantitative study of renin in the different layers of the rabbit uterus. Acta path microbiol scand *67* 169–179 1966

3 *Christlieb A R Couch N P, Amsterdam E A Dobrinsky S J & Hickler R B* Renin extraction by the human liver Proc Soc Exp Biol Med *128* 821–823 1968

4 *Eskildsen P C* Place of renin formation in rabbit uterus Acta path microbiol scand Section A *78* 473–481 1970

5 *Eskildsen P C* Location of renin in rabbit uterus by help of microdissection Acta path. microbiol scand Section A *80* 241 252 1972[a]

6 *Eskildsen P C* Evidence for local renin formation in the rabbit oviduct Acta path microbiol scand Section A *80* 743 750, 1972[b]

In the study reported here the ditetrazol salt N BT (Sigma) has been used in the dehydrogenase reactions Spectrophotometric analysis of the reduction products has given absorption graphs which correspond to those described by *Gabler et al* (6)

An account is given of the incidence of half-formazans in a material comprising 200 biopsy specimens of human breast tissue There is also a discussion of whether HF may influence the evaluation of the enzyme histochemical reactions

The present paper deals solely with the incidence of HF For details about localisation and intensity of the activities of the individual dehydrogenases reference is made to a previous paper by the author (8) among others.

MATERIAL

The material comprised 200 biopsy specimens of breast tissue from women admitted for operation for breast tumour (194 biopsies) or mammary hypertrophy (6 biopsies) In the first named 194 cases the histological diagnoses were as follows Fibroadenomatosis in 98 cases, fibrosis in 6 fibroadenoma in 8 carcinoma *in situ* in 7 and carcinoma in 75 In the 6 cases with a diagnosis of mammary hypertrophy microscopy revealed nothing abnormal in 2 while in the remaining 4 cases biopsy disclosed very mild forms of fibrosis or fibroadenomatosis

The biopsy specimens were taken during operation and immediately frozen down to -80° C in an acetone/solid CO_2/isopentane freezing mixture They were then stored at 20° C until the enzyme histochemical estimations were made

TECHNIQUE

Freshly frozen tissue specimens were sliced on a cryostat (SLEE, Pearse) in 8 μ thick sections after which the following reactions were used for the demonstration of dehydrogenase activity NADH tetrazolium reductase (NADH Tr) NADPH tetrazolium reductase (NADPH Tr) lactic acid dehydrogenase (LDH) succinic acid dehydrogenase (SHD) glucose 6 phosphate dehydrogenase (G 6 PD) α glycerophosphate dehydrogenase (α GPDH) and alcohol dehydrogenase (ADH) The staining methods described by *Thomas & Pearse* (22) were used All the tests were supplemented by control tests consisting of incubation of tissue sections in incubation fluids without addition of the specific substrates Some of the biopsy specimens were also used in colour reaction tests for demonstration of SH groups using the technique described by *Bennett* (3) Haematoxylin eosin stained tissue sections were used as aids in the histological assessment The anaplasia of the carcinomas was graded by the method devised by *Bloom & Richardson* (4)

RESULTS

Table I contains a survey of the results obtained in biopsies of the 200 specimens of breast tissue One hundred and eighteen biopsy specimens showed benign changes 7 carcinoma *in situ* and 75 manifest carcinoma HF was found in 16 of the 98 specimens with fibroadenomatosis, while 82 specimens did not show this double colouration of the reduction



Fig 1 Incidence of half formazans in tumour tissue during demonstration of NADH Tr activity The reduction products consisting of a mixture of blue d formazans and rose violet half formazans are seen to be irregularly localized in the tumour cells. Magnification × 80

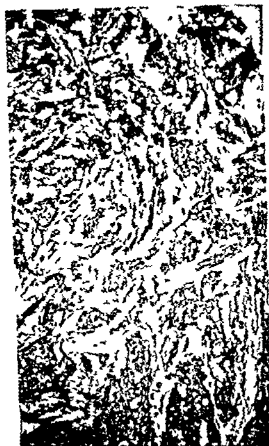


Fig 2 NADH Tr activity in tumour tissue without production of half formazans The reduction product consists of diformazans and is evenly distributed within the tumour cells Magnification × 80

product The next four groups in the table were too small to permit the drawing of any conclusions It is however noteworthy that neither the biopsy specimens showing fibrosis nor those with mammary hypertrophy contained HF HF was observed in the two out of six cases of fibroadenoma and in four out of seven cases of carcinoma *in situ* Within the group of carcinomas HF was present in 56 out of 75 biopsy specimens On classifying the carcinomas according to the degree of anaplasia the HF content was seen to increase with increasing anaplasia from 60 per cent for grade I to 89 per cent for grade III Figs 1 and 2 illustrate tumour tissue with and without HF production

The two large groups from Table 1 have been set out in Table 2 The groups comprised 75 cases of carcinoma and 98 of fibroadenomatosis In the column on the extreme right is indicated whether the control specimens (i.e tissue sections incubated without the specific substrate) showed positive reaction

In the group of carcinomas 28 specimens showed positive control reactions, while 47 did not In the group of fibroadenomatosis the corresponding figures were 42 positive and 56 negative control reactions It may be seen from the table that the presence of HF was not correlated to an increased incidence of positive reactions in the control sections

Table 3 shows the results of the tests for SH group activity Thirty six biopsy specimens with carcinoma were tested The staining method was not employed consistently for all the specimens, because this method is less suitable for freshly frozen tissue sections It was therefore necessary to transfer the frozen tissue blocks *via* formalin fixation to paraffin This precluded further testing for dehydrogenase reactions in these specimens In addition to the 36 carcinoma specimens set out in Table 3 a series of specimens with fibroadenomatosis were stained by this method, all with results corresponding to those for carcinoma Many biopsy specimens with carcinoma also contain circumjacent structures with fibroadenomatosis Benign and malignant structures could thus be compared in the same specimen The SH group activities were identical in benign and malignant tissue As HF is as stated produced mainly in the malignant tissue it was in the opinion of the author adequate to limit the evaluation of the SH reaction to the group with carcinoma in order to determine whether the SH groups might lead to production of HF (see discussion)

It can be seen from Table 3 that ten biopsy specimens with HF production had positive control specimens (C+) In five of these specimens SH group activity was found to be absent or very slight (0 and 0–1) whereas the remaining five showed activity of varying

TABLE 1 *Incidence of Half Formazans in Relation to Demonstration of Dehydrogenase Activity in Mammary Tissue*

	+ half formazans	– half formazans	Total number
Fibroadenomatosis	16 (16 %)	82 (84 %)	98
Fibrosis	0	6	6
Hypertrophy	0	6	6
Fibroadenoma	2	6	8
Carc *in situ*	4	3	7
Gr I	6 (60 %)	4 (40 %)	10
Carcinoma Gr II	34 (72 %)	13 (28 %)	47
Gr III	16 (89 %)	2 (11 %)	18
Total	78	122	200

TABLE 2 *Relation between Occurrence of Half Formazans and Positive Reaction in the Control Specimens during Demonstration of Dehydrogenase Activity*

	+ half formazans	– half formazans	Controls
Carcinoma	22	6	positive
	34	13	negative
Fibroadenomatosis	7	35	positive
	9	47	negative
Total	173		

TABLE 3 *Relation between Incidence of SH Groups Half formazans and Positive Control Specimens*

		HF +	HF
SH activity 0	C +	2	2
	C	6	4
SH activity 0 1	C +	3	2
	C	1	1
SH activity 1	C +	3	0
	C	3	1
SH activity 1 2	C +	1	0
	C –	3	2
SH activity 2	C +	0	0
	C –	1	0
SH activity 2 3	C +	1	0
	C	0	0
Total number of specimens 36		24	12

Abbreviations Half formazans HF Positive or negative control specimens C + or C

intensity The remaining 14 specimens out of a total of 24 with HF production had negative control specimens (C) and varying activities of SH groups

Testing for alcohol dehydrogenase activity revealed no or only sporadic reaction This could not be correlated to the incidence of SH groups nor to that of positive control specimens

DISCUSSION

The factors which may possibly contribute towards production of half formazans can be divided into two groups non enzymatic and enzymatic

Non enzymatic factors Contamination by admixture of monotetrazolium salts with the ditetrazolium salt used (N BT) is not improbable Reduction involves production of monoformazans which have a somewhat dif

ferent colour from the diformazans As stated in the introduction, such contamination can be excluded by spectrophotometry (6)

A direct reduction of the tetrazolium salt may occur in connection with increases in the pH of the incubation fluid (17) Checking the pH values before and after the incubation leads to the exclusion of this possibility as the cause of HF production

Exposure to coenzyme accelerators or substitutes, such as phenazine metosulphate, could be excluded in the present study, as no such subsidiary substances were used

The question of the lipid solubility of the formazan compounds must be approached with caution Thus *Kahan* (11) has reported that in experimentally produced degenerative myocardial changes with lipid precipitates there were alterations in the ability of N BT to form differently coloured reduction products

While the diformazans which are formed by the reduction of N-BT would seem to be completely lipid insoluble (15), it is to be expected that the monoformazans and half-formazans would show a varying degree of lipid solubility with a resultant change in the localization of the reduction products in the various types of tissue

In the present study HF was found to be localized exclusively to the epithelial elements (epithelial cells in the exocrine ducts and the tumour cells) The fat cells were unstained apart from a narrow rim of blue granules along the cell membrane indicating the deposition of diformazan in the aqueous phase

The author considers that the *formation* of HF cannot be explained merely as the result of a formazan lipid interaction There are only small amounts of fat in the tumour tissue and there is no correlation between the amount of fat and the degree of anaplasia of the tumour In contrast HF is found in amounts which obviously increase with increasing degree of anaplasia and thus must be assumed to reflect a correlation with the tumour tissue

The SH groups present in the tissue sections may cause an indirect reduction of N-BT through an antecedent reduction of the coenzyme which is added to the incubation fluid for demonstration of the activity of the coenzyme-dependent dehydrogenase Such a non enzymatic reduction has often been described in the literature as a "nothing dehydrogenase reaction" (1) (7) (9) (16) (20) (21) (23)

The activities of the following coenzyme-dependent dehydrogenases were studied as part of the present investigation LDH, G 6-PD, α-GPDH, and ADH In addition, SDH was studied as a representative of the coenzyme independent dehydrogenases, and, finally, the activities of NADH-Tr and NADPH-Tr were demonstrated The last two enzyme systems involve the reduced coenzymes NADH and NADPH as specific substrates, and the reaction is not influenced by any SH-groups which may be present

If the SH-groups were responsible for the production of HF, the presence of HF would always be associated with positive control specimens This is not the case (Table 3) Further, HF would only be found in relation to demonstration in the presence of coenzyme-dependent dehydrogenase activity This is not in accordance with the results of the present investigation, which show that HF may occur in association with any dehydrogenase reaction The presence of HF was in fact most pronounced in tests for activity of NADH-Tr and NADPH Tr, in which the SH groups could have no influence An SH group reaction can thus be excluded

The enzymatic factors may be represented *partly by the above-mentioned specific dehy*drogenases and partly by the ADH present in the tissues, which can reduce the coenzymes and thus contribute towards a false positive control reaction However, the influence of ADH can only lead to HF production where there is activity of coenzyme dependent dehydrogenases, and this can therefore be excluded, as HF can be demonstrated in all dehydrogenase reactions The ADH activity was tested in 95 biopsy specimens (48 malignant and 47 benign) In by far the majority of cases there was little activity, and ADH

activity may thus be excluded as a possible cause

The author therefore considers that the HF production is a direct consequence of the activity of the specific dehydrogenases, because a varying intensity of the enzyme activities may be assumed to lead to the production of varying amounts of incomplete reduction products, i e HF

As shown in Table 1, HF production was seen particularly in the biopsy specimens with carcinoma Table 2 illustrates that there is a correlation between the degree af anaplasia of the carcinomas and the incidence of HF in the tumour cells This must presumably be regarded as evidence of alterations in the tumour cell enzyme activity HF production may thus perhaps be useful in the assessment of the oxidative metabolism of the tumour tissue, instead of being considered merely as an undesirable staining, as has hitherto been the case

As shown in Tables 1 and 2 HF was found in a small number of biopsy specimens from cases of fibroadenomatosis The significance of the HF production in these benign disorders could probably be elucidated by following up this group of patients

---

The work was aided by grants from *Daell fonden* and from *Landsforeningen til kræftens bekæmpelse*

REFERENCES

1 *Andersen, H* Sulphydryl nothing dehydrogenase activity and lactic dehydrogenase $NADH_2$ cytochrome C reductase reactions in tissues of human foetus Acta Hist (Jena) *21* 120–134 1965

2 *Barka, T & Anderson, P J* Histochemistry Theory practice and bibliography Hoeber Medical Division New York Evanstone and London 1963 p 300

3 *Bennett H S* Cytochemical evidence for sulphydryl in nerve axoplasm retinal rods and capillary endothelium Anat Rec *100* 640 1948

4 *Bloom, H J & Richardson B W* Histological grading and prognosis in breast cancer Brit. J Can *11* 359 377, 1957

5 *Burstone, M S* Enzyme histochemistry and its application in the study of neoplasms Academic Press New York and London 1962 p 483

6 *Gabler, W, Wendler, D & Schmidt, B* Chromatographische und Spectrophotometrische Untersuchungen an Nitro-BT und seinen Formazanen Acta Hist (Jena) *36* 295–302 1970

7 *Hoyer, P F & Andersen, H* Specificity in steroid histochemistry, with special reference to use of steroid solvents. Histochemie *24* 292–306, 1970

8 *Jensen, H* Fibroadenomatosis and breast carcinoma Enzyme histochemical studies Acta path microbiol scand Section A *78* 421 431, 1970

9 *Jensen, H* Quantitative determination of dehydrogenase activity in tissue sections and tissue homogenates. Acta path microb ol scand Section A *80* 548 556, 1972

10 *Jones, G R N* The purification and some properties of neotetrazolium cloride and its chief monotetrazolium salt contaminant Histochem J *1* 59 67, 1968

11 *Aakari, S* Observations on the use of nitro blue tetrazolium in the detection of early myocardial changes Histochem J *2* 453–477 1970

12 *Nachlas, M M Tsou K C Sou a, E Cheng C S & Seligman, A M* Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole J Hist Cytochem *5* 420–436 1957

13 *Nachlas M M Walker D G & Seligman A M* A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase J biophys. biochem cytol *4* 29–36 1958

14 *Pearse A G E* Extension of the limits of cellular pathology The role of enzyme histochemistry J clin. path *11* 520 534 1958

15 *Pearse A G E* Histochemistry theoretical and applied vol 2 3rd ed Churchill Livingstone Edinburgh and London 1972 pp 890–899

16 *Racker E* Mechanism of action and properties of pyridine nucleotide linked enzymes. Phys rew *35* 1–50 1955

17 *Rust, J B* Chemical properties of the tetrazolium salts. Trans N Y Acad Sci *17* 379–384 1955

18 *Rutenburg A M Golfstein R & Seligman A M* Preparation of a new tetrazolium salt which yields a blue pigment on reduction and its use in the demonstration of enzymes in normal and neoplastic tissues Cancer res *10* 113–121 1950

19 *Seidler E* Über die Ursachen der Verschiedenfarbigkeit der Reaktionsprodukte histoche

mischer Tetrazol Techniken Acta hist (Jena) 32 428–431, 1969
20 *Shaw, C R & Koen, A L* On the identity of nothingdehydrogenase J histochem. *13* 431–433 1965
21 *Stiller D & Hempel, E* Zum Problem des nothingdehydrogenase Effektes beim topochemischen Nachweis dehydrierender Enzymsysteme. Acta Histochem. (Jena) *35* 239–252, 1970
22 *Thomas, E & Pearse, A G E* The fine localization of dehydrogenases in the nervous system Histochemie 2 266–282, 1961
23 *Zimmerman H & Pearse, A G E* Limitations in the histochemical demonstration of pyridine nucleotidelinked dehydrogenases J histochem cytochem. *7* 271–275, 1959

Acta path microbiol scand Section A 81,3, 276-280, 1973

PULMONARY BONE MARROW EMBOLISM

A Histological Study of a Non-selected Autopsy Material

Ö Haug and O P N Gruner

The Ullevål Hospital, Department of Pathology,
Head Professor Kristen Arnesen M D

Bone marrow embolism (BME) was found in 80 out of 507 consecutive autopsies (15 7 per cent) BME was associated with accident in more than 50 per cent and with cardiac disease in approximately 25 per cent of the cases. Mechanisms for the production of BME other than fracture must be sought since 8 per cent of the cases did not have any bone involvement BME should not be considered proof of antecedent intravital trauma and pulmonary BME did not appear to have any clinical significance

Bone marrow embolism (BME) is apparently an infrequent condition possibly because it may be overlooked (*Fisher* 1951) It is commonly assumed (*Palmovic & McCarrol* 1965, *Mason* 1968, *Bhashkaran* 1969) that bone involvement (fracture or orthopaedic operation) is a prerequisite Cases of pulmonary BME without visible fracture have been reported, however (*Rappaport et al* 1951 *Yanoff* 1963, *Winkel & Brown* 1961 *Koblich & Kreiner* 1969)

According to *Fisher* (1951), pulmonary bone marrow emboli appear perfectly bland i e there is no vessel wall reaction at the point of arrest nor pulmonary infarction Nevertheless it has been commonly assumed that pulmonary BME may be lethal if emboli are multiple (*Rappaport et al* 1951, *Zichner* 1970) Systemic BME which requires an open foramen ovale has, on the other hand, been associated with minute infarctions of the brain and the kidneys (*Ghatak & Zimmerman* 1971)

Neither incidence pathogenesis nor importance of pulmonary BME seem clear The present paper is a prospective study of a non selected standardized autopsy material Previous reports have, to the knowledge of the authors failed to fulfil one or more of these criteria.

MATERIAL

Among 2820 consecutive autopsies 508 were randomly allotted for the present study *Age* and *sex* distribution is given in Table 1 and 2

All a[illegible]topsies were performed at the Department [illegible]

an all white population of 490 000 About 80 per cent of deaths in the Hospital are examined post mortem. Social and economic factors probably play a minimal role in the selection of cases as the cost of hospital care is met by compulsory national health insurance

METHODS

Autopsy was carried out 8 to 18 hours after death and in the majority of cases within 24 hours Four specimens one from the surface of the upper and lower lobe of each lung were fixed in 4 per cent formaldehyde solution embedded in paraffin and

---

Received 15 x 72 Accepted 29 xi 72

Requests for reprints should be addressed to Dr *Ö Haug*, Patologisk anatomisk lab Ullevål Sykehus, Oslo 1, Norway

ital records

Statistical methods The histological sections were examined without access to the autopsy number or the case report and the code was not broken until the complete material had been examined (double blind technique) Inter group differences (two-by two tables) were compared with the Fisher Irwin test (two-tailed), correlations described by the Spearman rank correlation (R) and confidence intervals given with 95 per cent limits

RESULTS

BME was found in 80 cases, i e in 15 7 per cent (12 9 19 5 confidence interval) The incidence decreased significantly with age (Table 1 and Fig 1) and was found to be highest among males (Table 1)

TABLE 1 *BME According to Age and Sex*

Years	Males	Males with BME	Females	Females with BME
30–39	6	2	3	1
40–49	21	4	9	1
50–59	39	8	25	4
60–69	86	19	52	3
70 79	89	15	82	11
80–89	31	4	49	7
90–	7	0	9	1
	279	52*	229	28*
		18 6 %		12 7 %

* P ~ 0 06

TABLE 2 *BME According to Underlying Cause of Death*

	Number of cases	BME	%
Accident	28	15	53 6
Postoperative state	36	7	24 1
Cardiac disease	162	38	23 5
Cerebrovascular disease	61	3	4 9
Malignant tumour	135	6	4 4
Gastro-intest disease	23	3	13 0
Miscellaneous	63	8	12 7
Total	508	80	15 7

TABLE 3 *BME According to Immediate Cause of Death*

	Number of cases	BME	%
Accident	25	14	56 0
Postoperative state	10	1	10 0
Cardiac disease	131	40	30 5
Cerebrovascular disease	43	2	4 7
Malignant tumour	45	6	13 3
Pulmonary embolism	91	13	14 3
Pneumonia & aspiration	96	1	1 0
Miscellaneous	67	3	4 5
Total	508	80	15 7

TABLE 4 *BME According to Bone Involvement*

	Number of cases	BME	%
Bone involvement	97	46	47 4
No bone involvement	411	34	8 3
Total	508	80	15 7

P = 0 003

Cause of death (Table 2 and 3) More than half of the accident cases were associated with BME Cardiac disease also appeared to be connected with BME All accidents but one included fracture and costal fracture after external cardiac massage occurred in most of the cardiac cases Cerebrovascular disease and malignant tumour were seldomly associated with BME

Bone involvement (Table 4), whether due to major fracture, orthopaedic operation, or costal fracture after cardiac massage, was associated with BME in nearly 50 per cent of the cases as compared with 8 3 per cent (5 8–11 5 confidence interval) in the rest of the cases

There was seldom more than one embolus in each *section* and BME was most often found in one section only (Fig 2) Bone involvement did not significantly affect this distribution

BME was most often observed subpleurally The emboli did not provoke tissue reaction

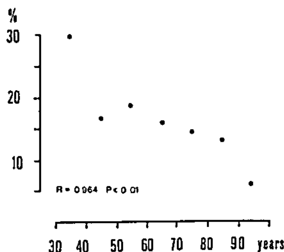


Fig 1 Percentage of cases with BME in each decade

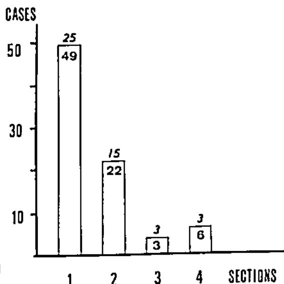


Fig 2 Cases with BME in 1 2 3, and all 4 sections respectively Cases with bone involvement in *italics*

(Fig 3 and 4) In one case, bone marrow was found within a thrombo-embolus Otherwise fibrin deposition was not associated with BME

DISCUSSION

The present number of cases with pulmonary BME exceeds, to the knowledge of the authors, the number seen in any previous material The incidence of 15 7 per cent may seem to be in contrast with the observation by *Rappaport et al* (1951) who did not find a single conclusive case in a series of 240 consecutive autopsies Death due to accident, however, was infrequent in their material and it is not stated whether the latter had been examined 'blindly' Furthermore, the number of pulmonary sections studied in each case was inconstant *Zichner* (1970) found a BME incidence of 36 6 per cent in a series of selected fracture cases while *Rappaport et al* (1951) in a similar group found 6 per cent BME

The incidence of BME decreased moderately with age This may possibly be due to the fact that red marrow is replaced by yellow marrow and that the latter could not be recognized as bone marrow in the sections It is

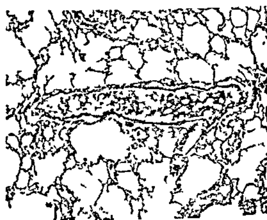


Fig 3 Bone marrow embolus in lung artery Lendrum

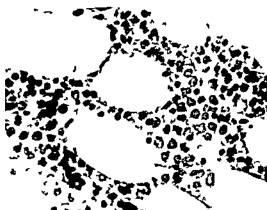


Fig 4 Large magnification shows fat cells and haematopoetic tissue Lendrum

also possible that red marrow is more fragile than yellow, at least it is more vascular and bone marrow haemorrhage seems to be causally related to BME (*Ryzlin et al* 1963) On the other hand, the age as well as the sex difference may also be ascribed to a preponderance of accident as the cause of death in the younger age groups and among males

The present observations seem to confirm the generally assumed association between BME and violent death (*Mason* 1968 *Bhaskaran* 1969 *Zichner* 1970) BME and cardiac death were also associated Most of these cases had been subjected to cardiac massage and costal fracture had occurred It seems however as if cardiac massage may be associated with BME whether or not costal fracture is inflicted (*Winkel* & *Brown* 1961 *Yanoff* 1962)

BME and bone involvement were significantly associated a fact which apparently is in agreement with the concept that traumatic disruption of bone marrow is a prerequisite for BME (*Rappaport et al* 1951 *Mason* 1968) However BME was also found even though there was no fracture or other bone involvement Minute fractures may admittedly have been overlooked in some of the cases although the ribs were scrutinized and the vertebral corpora chiseled open in each case On the other hand a number of other reports of BME without bone involvement are available (*Rappaport et al* 1951 (review) *DeLand* & *Bennet* 1957, *Garvey* & *Zak* 1964 *Rogel et al* 1965 *Koblich* & *Kretner* 1969) and the common deduction that BME is proof of an otherwise ignored fracture may seem presumptuous It is therefore considered probable that BME as in laboratory animals (*Ogata* 1912) may be a normal event i e an incidental finding which is exaggerated in accidents (mainly major fractures) and in cardiac disease Both are conditions from which young and otherwise healthy people may die suddenly

The present distribution of bone marrow emboli on sections indicates that bone involvement does not significantly affect the extent of BME The distribution seems to support the observation by *Fisher* (1951) and *Mason* (1968) that the incidence of BME will increase if sections to be examined in each case are more numerous

Therefore it seems to be a matter of pathogenic mechanisms in addition to fracture, or perhaps instead of fracture, i e that fracture may be only indirectly related to BME Haemorrhage and ischaemia of the bone marrow are thus associated with BME (*Ryzlin et al* 1963 *Mason* 1968) and may perhaps explain the occurrence of BME after accidents and cardiac infarction as bone marrow pressure is substantially reduced in low flow states (haemorrhagic shock) (*Kitka et al* 1972) and in other stress situations including catecholaminaemia (*Stein et al* 1958) The release of immature cells such as megakaryocytes following trauma (*Breslow et al* 1968) may serve as a comparable phenomenon (*Lengemann* 1899)

Convulsions especially in young people, have been associated with BME (*Rappaport et al* 1951) Bony concussion (jarring) or minute indetectable medullary fractures have been considered involved in the mechanism (*Rappaport et al* 1951 *Bhashkaran* 1969) How often this common agonal phenomenon had occurred in the present material could not be ascertained Irrespective of an uncertain pathogenesis the fact that BME may occur without antecedent trauma indicates that it no longer can be considered of medicolegal consequence

The bone marrow emboli were usually found in subpleural vessels This contrasts with the observation by *Mason* (1958), who did not find any definite distributional pattern The emboli appeared perfectly bland, as previously described by *Fisher* (1951) and were in no case considered of lethal significance In contrast to *Zichner* (1951) we did not observe breakdown changes of the emboli or granuloma formation Consequently it also seems doubtful whether BME is of clinical significance

---

This work was supported by a grant from "*Nasjonalforeningen Det norske hjerte og karråd*"

REFERENCES

Bhaskaran, C S Bone marrow embolism in traumatic death Path Microbiol *34* 105 111 1969

Breslow, A, Kaufman, R M & Lawsky, A R The effect of surgery on the concentration of circulating megakaryocytes and platelets *Blood 32* 393–401, 1968

DeLand, F H & Bennett, W A Death due to bone marrow and tumor embolization in the abscence of fracture *Arch Path 63* 13–16, 1957

Fisher, J H Bone marrow embolism Arch Path *52* 315 320, 1951

Garvey, J W & Zak, F G Bone marrow emboli from external cardiac massage J Amer med Ass. *187* 59 60, 1964

Ghatak, N R & Zimmerman H M Cerebral bone marrow embolism Arch *Path 92* 112–118 1971

Kitka, R, Witoslawa M M, Hopkins R W & Simeone, F A Bone marrow pressure and blood flow and resistance in experimental hemorrhagic shock Amer J Surg *123* 380–384, 1972

Koblich, R & Kreiner, E Bone marrow embolism in multiple myeloma Virch Arch Path Anat *347* 153–159 1969

Lendrum, A C, Fraser, D S Slidders W & Henderson R Studies on the character and staining of fibrin J clin Path *15* 401 413 1962

Lengemann P Knochenmarksveränderungen als Grundlage von Leukocytose und Riesenkern verschleppungen (Myeolokinese) Beitr path. Anat *29* 1–40, 1901

Mason, J K Pulmonary fat and bone marrow embolism as an indication of ante mortem violence Med Sci Law *8* 200–206, 1968

Ogata, S Megakaryocytenembolie und Knochenmarksembolie in Lungenkapillaren Beitr path. Anat *53* 120–128 1912

Palmovic, V & McCarrol, J R Fat embolism in trauma Arch Path *80* 630–635 1965

Rappaport, H, Raum, M & Horrell J B Bone marrow embolism Amer J Path *27* 407–433 1951

Rogel S, Rosenmann E & Rachmilewitz E A Multiple pulmonary infarctions caused by bone marrow embolism New Engl J Med *272* 732–734, 1965

Ryalin A M, Block, A L & Werner, C S Hemoglobin C & S disease in pregnancy *Amer J* Obstet Gynec *86* 1055–1059 1963

Stein A H Morgan, H C & Porras R F The effect of pressor and depressor drugs on intramedullary bone-marrow pressure *J Bone Joint* Surg *40* A 1103 1110 1958

Winkel F C & Brown, W C Bone marrow embolism following closed chest cardiac massage *J* Amer med. Ass *178 329 331 1961*

Yanoff, M Incidence of bone marrow embolism due to closed chest cardiac massage New Engl J Med *269* 837 839 1963

Zichner L Zur Bedeutung der Spongiosa und Knochenmarksembolie in die Lunge Langenbecks Arch Chir *326* 367 379 1970

Acta path microbiol scand Section B 81, 3 281-290, 1973

ULTRASTRUCTURE OF A MEASLES-CARRIER HUMAN CELL LINE

W K. HENEEN, A LEVAN and W W NICHOLS

Institute of Genetics Lund Sweden, and
Institute for Medical Research, Camden N J USA

Electron microscopic studies were conducted on cultured monolayers of human cells (LU 106) persistently infected with the Edmonston strain of measles virus Viral nucleocapsids were detectable in the cytoplasm and less often in the nucleus. The aspects of virus/cell interrelationships described and discussed are (a) the occurrence of viral materials in metaphase cells (b) the appearance of degenerating cytoplasms and nuclei, (c) the occurrence of microtubules in cellular projections that contained aligned nucleocapsids and (d) the release of nucleocapsids through the rupture of the cell membrane

In addition to interest in clinical measles there has been considerable speculation about the role of measles virus or another closely related virus in the aetiology of certain diseases of the central nervous system in man (e g 26 32) The fine structure of measles virus infection has been studied in cultured cells of human (12 14 16 20 22 34) African green monkey (1 2 15 17 22 26) and dog origins (33) as well as in cells from the nervous system in mice (38) and hamsters (3 27, 28) To our knowledge no fine structure studies were made on cells that are persistently infected with measles virus

A cell line which is a carrier for measles virus has been established by Norrby (20) from the human heteroploid cell line LU 106 The culture represents a balanced condition of virus activity and cell growth In a cell population of this culture one finds different intensities of infection all stages of virus replication and production and different stages of cell degeneration The present work is mainly concerned with some ultrastructural features of virus/cell interrelationships in this culture

Received 31 x 72 Accepted 12 xii 72

MATERIALS AND METHODS

The cell line studied is a carrier of measles virus and was kindly supplied by Dr *Erling Norrby* The carrier cell line has been established by *Norrby* (20) from the heteroploid human cell line LU 106 which was exposed to the Edmonston strain of measles virus LU 106 originated from a human male embryo and is near triploid with a stemline number of 69 70 chromosomes A lower stemline number of 66 68 has been recorded for the carrier cells (19)

The carrier state implies the maintenance of cell growth in spite of viral replication If the usual amount of culture medium (10 ml/milk dilution bottle) were used cell destruction due to viral activity would sometimes be pronounced and endanger the viability of the cells The cells were therefore maintained in three times as much as the usual amount of medium. Under this condition cell growth was sufficient to maintain the culture The carrier cells were grown in Hank s medium supplemented with 10 per cent calf serum. At the time the present study was made the culture was found to be positive for mycoplasma This was taken into consideration when the findings in these cells were interpreted

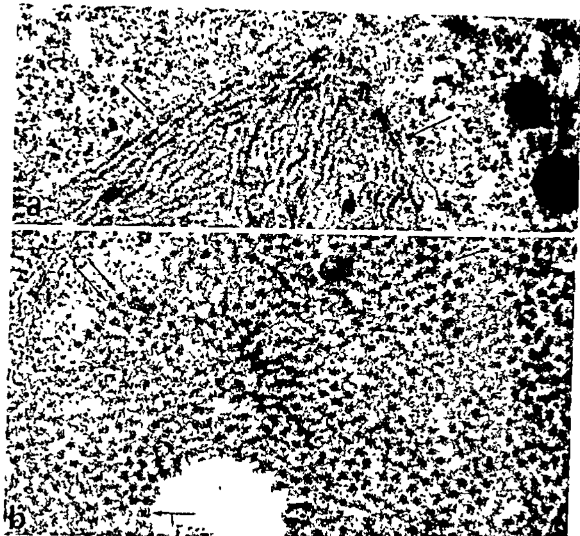


Fig 1 Fuzzy viral nucleocapsids in the cytoplasm a Part of a patch (limited by arrows) of nucleocapsids at low magnification X 19,000 b Nucleocapsids cut *mainly transversely (arrows)* X 47,000

For electron microscopy, monolayers grown in Falcon bottles were fixed and embedded in situ according to the method of *Brinkley et al* (5) Polykaryocytes with interphase nuclei, and mitotic figures representing division in polyploid or multinucleated cells were chosen for further sectioning using an LKB ultramicrotome Silver sections were stained with uranyl acetate and lead citrate and observed in a Hitachi HS 7S electron microscope at 50 KV

RESULTS

Different stages of viral development as well as various degrees of cellular infection and degeneration were present in the same culture bottle of these permanently infected cells Such a system is thus not suitable for a study of the sequence of events following infection In view of the extensive knowledge of measles virus infection on an ultrastructural basis (1-3, 16, 17, 27, 28), the present observations will be mainly focused on aspects that have not been described before and aspects that have some bearing on earlier reported findings

Cytoplasmic Aspects

Materials that represented measles virus nucleocapsids were of common occurrence in

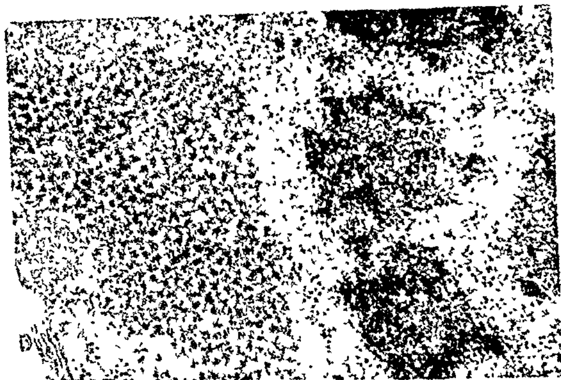


Fig 2 Nucleocapsids in a metaphase cell The nucleocapsids are in the left half of the picture, whereas the electron dense materials to the right are parts of chromosomes. X 47 000

the carrier cells (Fig 1) but they were absent in LU 106 cells These viral materials were observed in mono-, bi and multinucleated cells The later types of cells were frequently found in the carrier cell line A characteristic feature of measles virus is its ability to induce polykaryocytosis (reviewed in 11) The frequency of occurrence and the amounts of the viral materials were much higher in the cytoplasm than in the nucleus The nucleocapsids appeared as arrays of filamentous materials The tubular component in the middle of the filamentous structure is clearly seen in transverse sections (Fig 1b) The diameter of this tubule-like component is about 17 nm, whereas the diameter of the filament is about 45 nm The nucleocapsid masses represent a stage of viral development or a viral condition which is easy to detect morphologically Cytoplasmic materials and patches that possibly represent earlier stages in viral development cannot be identified with certainty

Of interest here is the observation that cells containing viral nucleocapsids are capable of entering into mitosis Part of a cell containing condensed chromosomal material most likely at metaphase, is shown in Fig 2 The easily detectable viral nucleocapsids occur in patches at the periphery of the cell If virus materials at other stages of organization occur close to or within the chromatin they will be difficult to ascertain

In degenerating cells the cytoplasm loses its integrity and the cytoplasmic contents appear as rounded patches or vesicles of materials that vary in electron density (Fig 3) Viral nucleocapsids are conspicuous in Fig 3a whereas in Fig 3b tubular and nucleocapsid like structures are revealed in the cytoplasmic islets suggesting their viral nature

Nuclear Aspects

The occurrence of detectable viral materials in the nucleus is much less frequent than

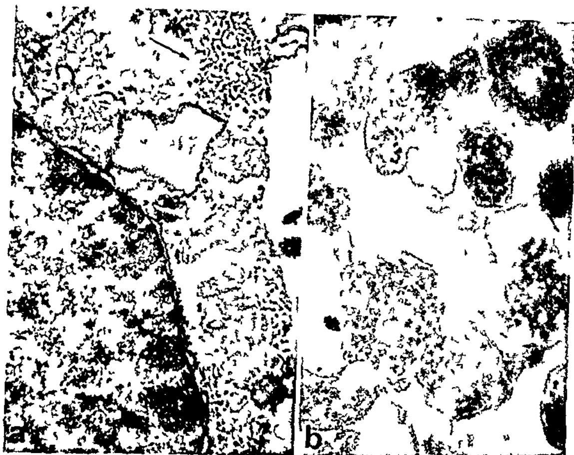


Fig 3 Viral materials in the cytoplasm of degenerating cells a Nucleocapsids (arrow) × 17 500 b Islets of nucleocapsid like materials × 47 000

in the cytoplasm Nuclei with nucleocapsids were found in cells whose cytoplasm usually contained large patches of viral materials Affected nuclei were also seen in cytoplasms that appeared normal This was recorded in single sections Serial sectioning would be needed in order to determine the absence or presence of viral materials in the cytoplasm of the cell in its entirety

The nuclear material in degenerating cells exhibited a variety of morphological features In Fig 4a materials having at least five different textures can be differentiated in the nucleus of a degenerating cell Higher magnifications of four of these materials are shown in Fig 4b The electrondense material (A) lining the nuclear membrane evidently represents condensed chromatin The least condensed patches (B) might also represent diffuse chromatin materials inside the nucleus The patch of tubular material (E) seems to be tubular components of viral nucleocapsids These tubules have the same diameter as those found in the cytoplasm They seem *however to lack the enveloping fuzzy* materials seen in the cytoplasmic nucleocapsids The nucleolus (C) is uniform in texture Dispersed electron dense materials (D) that give the impression of being pyknotic can be found associated with or in the vicinity of the nucleolus Whether this material represents pyknotic chromatin a segregated nucleolar component or a viral substance remains to be determined

Processes at the Cell Membrane

Extrusion of viral materials from cells was mainly observed through the alignment of nu

cleocapsids underneath the cell membrane mainly in finger-like cellular projections accompanied by the development of materials at the outer edge of the plasma membrane opposite these nucleocapsids, and the subsequent pinching off of membraneous and cytoplasmic materials A zipper like arrangement of nucleocapsids along parallel membranes was sometimes noticeable Microtubules about 20 nm in diameter usually occurred in the cellular projections (Fig 5) The function of these microtubules might be to achieve rigidity for the cellular processes

Free nucleocapsids in the vicinity of ruptured cell surfaces were observed in a few cells (Fig 6) The appearance of the cytoplasm and the nuclei in these cells did not imply that they were in a degenerating condition Large amounts of fibrous and microtubular materials were found in the cytoplasm behind the extruded viral nucleocapsids The maximum length of nucleocapsids was about 1 µm The characteristic herringbone like appearance of the tubular component can be discerned in the individual nucleocapsids

DISCUSSION

Persistent measles virus infection in vitro has been established in a few cases (7 20, 25, 30) In all these studies tissue cultured cells of human origin were used Besides the present cell line which is of an embryonal lung origin (20) a carrier state has been developed in HeLa (25 30) and amnion (7) cells Persistent infection with measles virus in mouse brain cell cultures infected *in vivo* has also been reported (10) That measles virus nucleocapsids were frequently encountered in the LU 106 carrier cells is in agreement with the finding that these cells are positive for viral properties such as haemolytic, haemagglutinating and complement fixing activities (20)

The ultrastructural features of the LU 106 carrier cell line are very much similar to those found in other cell systems following measles virus infection (1 3 16 17 27 28) These findings are also in agreement with the studies of this virus by fluorescence techniques (13, 21 29) The mechanism of viral budding depicted schematically by *Berkaloff* (4) for Sendai virus is most likely valid for other paramyxoviruses. The aspects of concern here are therefore those that are new or have some relevance to earlier observations Such aspects comprise (a) the occurrence of nucleocapsids in mitotic cells (b) the appearance of viral and cellular components in degenerating cells (c) the presence of microtubules in cellular projections that contain aligned nucleocapsids, and (d) the possibility that rupturing or disorganization of cell membranes may be a mechanism of nucleocapsid release

In agreement with earlier findings (3 27 28) is the observation that intranuclear nucleocapsids appear as smooth tubular structures about 17 nm in diameter whereas those in the cytoplasm have a diameter of about 45 nm due to the presence of fuzzy fibrous materials around the tubular component In the studies by *Nakai* and *Imagawa* (16) and *Nakai* et al (17) however both intranuclear and intracytoplasmic nucleocapsids were found to be smooth and to have a diameter of 15 18 nm The characteristic tubular appearance of nucleocapsids is the determining factor that a certain material is viral in nature The tubular appearance is most likely the ultimate stage in nucleocapsid development It is difficult to differentiate the early stages of viral development Apparently viral materials at these stages do not differ much in their appearance from normal cellular constituents

Of interest in the present study is the demonstration on an ultrastructural level that viral nucleocapsids occur in dividing cells Whether such cells represent an arrested condition or will progress normally through the later stages of mitosis is not known Apparently this will depend on the general condition of the cell and the intensity of viral infection Since the majority of the cells in the culture contain viral materials it is most likely that mitotic division is successfully accomplished in most cases That the carrier state in LU 106 cells is not the consequence of selection

A
E
D
B
a
B
A
D
E
b



Fig 5 The presence of microtubules in cell projections with nucleocapsids aligned under the cell membrane. a Nucleocapsids cut transversally b Nucleocapsids cut obliquely or *longitudinally* X 47 000

of resistant cells (20) also implies that infected cells are capable of propagating In accord with the present findings is the demonstration by fluorescence techniques that measles virus antigens occur in mitotic stages of HEp-2 cells not only at metaphase but also at later stages of the mitotic cycle (31) The present system seems to be the one suitable for a study of virus/chromosome interrelationships (18)

Fig 4 Nucleus of a degenerating cell a The differentiation of the nuclear contents into materials with different texture and electron density A condensed chromatin B uncondensed chromatin C nucleolar material D dispersed electron-dense materials E nucleocapsids X 17 500 b A high magnification of part of the same nucleus X 47 000

The frequent occurrence of degenerating cells allowed a detailed analysis of their fine structure Except for the presence of tubular nucleocapsids the majority of the other observed features in these cells are probably nonspecific changes That viral materials in some form other than tubular nucleocapsids are intermixed with specific cytoplasmic or nuclear constituents cannot be excluded

Cellular processes with aligned nucleocapsids contain microtubules that are about 20 nm in diameter which is slightly larger than the diameter of the viral tubular component Most likely these microtubules have a skeletal function in that they keep the cell processes rigid possibly also maintain them in special spatial interrelationships As demonstrated by *Baringer* and *Griffith* (3), and *Raine* et al



Fig 6 Ruptured cell membranes and release of *nucleocapsids*. Note the presence of fibrillar and microtubular material in the cytoplasm behind the released viral materials X 47,000

(27, 28), and observed here, viral materials *in different cell processes were usually in register with each other* The microtubular structures might also be functionally involved *in the process of viral extrusion*

Of interest is the observation of ruptured cell surfaces and nucleocapsids released from cells that did not show signs of degeneration It is known that cell membranes can be induced to rupture following an osmotic shock This has been used as a method by which virus materials might be extruded from cells. Thus a technical artifact could give rise to such a feature It should be pointed out, however, that the sectioned materials in the present study are cell monolayers that have been fixed in situ without any kind of pretreatment The significance of the presence of large amounts of fibrous and microtubular materials in the cytoplasm behind the extruded viral nucleocapsids and the connection of these materials with a possible mechanism of nucleocapsid release, remain to be elucidated

The maximum lengh of individual nucleocapsids that were found outside ruptured cells was about 1 μm This is in agreement with the average lengths reported for measles and other paramyxoviruses (8 9, 17, 23, 35)

Further relevant information on the nature of the measles virus and other myxoviruses is available elsewhere (6, 24, 36, 37)

Most likely, the viral materials outside the cells are to a great part the result of viral release from viable cells through budding or possible rupture of cell membranes Extra cellular viral materials could also have a cytoplasmic or nuclear origin, being a result of cell degeneration This should be taken into consideration in analyses of viral materials obtained through chemical extraction and centrifugation

The studied carrier cell line is a useful system for a further analysis of the state of a persistent infection with measles virus which has been suspected to be connected with the aetiology of certain nervous system diseases in man

---

The excellent technical assistance of Mrs. *Bodil Strombeck* and Mrs *Britt Lernius* is greatly acknowledged We sincerely thank Drs *Robert C Miller, Dan Moore Erling Norrby* and *Cedric C Raine* for fruitful discussions

This investigation was supported by grants from the *Swedish Cancer Society* and *The John and Augusta Persson Foundation* also grant No HD 06323 01 General Research Support grant FR 5 SO1 RR05582 and Research Career Award 5 K3 16 749 from the *U S Public Health Service* and Award No CA 11405 from the *National Cancer Institute*

REFERENCES

1 *Anismová E Mares I & Kyncl, F* Electron microscopic study of tissue culture cells infected with measles virus. Acta Virol *12* 289–295 1968

2 *Anismová E Mares I & Kyncl F* Electron microscopic study of cytoplasmic inclusions in cells infected with measles virus. Acta Virol *13* 257 263 1969

3 *Baringer J R & Griffith J F* Experimental measles virus encephalitis. A light phase fluorescence and electron microscopic study Lab Invest *23* 335–346 1970

4 *Berkaloff A* Étude au microscope électronique de la morphogenèse de la particule du virus Sendai J Microscop *2* 633–638 1963

5 *Brinkley B R Murphy, P & Richardson L C* Procedure for embedding in situ selected cells cultured in vitro J cell Biol *35* 279 283 1967

6 *Compans, R W, Mountcastle, W E & Choppin, P W* The sense of the helix of paramyxovirus nucleocapsids. J mol Biol *65* 167–169, 1972

7 *Enders, J R, Peebles, T C, McCarthy, K., Milovanović, M, Mitus, A & Holloway, A* Measles virus A summary of experiments concerned with isolation properties and behavior Amer J publ Health *47* 275–282, 1957

8 *Finch, J T & Gibbs, A J* Observations on the structure of the nucleocapsids of some paramyxoviruses J gen Virol *6* 141–150, 1969

9 *Finch J T & Gibbs, A J* The structure of the nucleocapsid filaments of the paramyxoviruses in *Barry R D & Mahy, B J* (Ed) The biology of Large RNA Viruses, Academic Press London & New York 1970 p 109–114

10 *Gibson P E & Bell T M* Persistent infection of measles virus in mouse brain cell cultures infected in vivo Arch ges. Virusforsch *37* 45–53 1972

11 *Heneen W K, Nichols W W, Levan, A & Norrby E* Polykaryocytosis and mitosis in a human cell line after treatment with measles virus. Hereditas *64* 53–84 1970

12 *Kallman, F Adams J M, Williams, R C & Imagawa D T* Fine structure of cellular inclusions in measles virus infections J Biophys biochem. Cytol. *6* 379 382 1959

13 *Llanes Rodas R & Liu C* A study of measles virus infection in tissue culture cells with particular reference to the development of intranuclear inclusion bodies J Immunol *95* 840–845 1965

14 *Mannweiler KI* Ultrastructural examinations of tissue cultures after infection with measles virus Arch ges Virusforsch *16* 89–96 1965

15 *Matsumoto N* Studies on measles virus in tissue culture (II) Electron microscopic study of measles virus infected cells and localization of virus antigen examined by Ferritin conjugated antibody method Bull Yamaguchi med School *13* 167 175 1966

16 *Nakai M & Imagawa D T* Electron microscopy of measles virus replication J virol *3* 187 197 1969

17 *Nakai T Shand F L & Howatson A F* Development of measles virus in vitro Virology *38* 50–67 1969

18 *Nichols W W* Relationships of viruses chromosomes and carcinogenesis. Hereditas *50* 53–80 1963

19 *Nichols W W Levan A Heneen W & Norrby E* Observations on a tissue culture cell line carrier of measles virus Fifth Conference on Mammalian Cytology and Somatic Cell Genetics. Nov 8 1966

20 *Norrby E* A carrier cell line of measles virus

in LU 106 cells Arch ges Virusforsch *20* 215-224 *1967*

21 *Norrby, E* Intracellular accumulation of measles virus nucleocapsid and envelope antigens. Microbios *5* 31-40, 1972

22 *Norrby, F, Chiarini, A & Marusyk, H* Measles virus variants, Intracellular appearance and biological characteristics of virus products. in *Barry, R D & Mahy, W J (Ed)* The Biology of Large RNA Viruses, Academic Press, London & New York 1970 p 141 152

23 *Norrby, E & Hammarskjold, B* Structural components of measles virus. Microbios *5* 17-29, 1972

24 *Norrby, E C J & Magnusson P* Some morphological characteristics of the internal component of measles virus. Arch ges Virusforsch *17* 443-447, 1965

25 *Oddo, F G & Tomasino, R M* Atti XII Congr *Naz* Microbiol, Perugia 1963, p 111

26 *Oyanagi, S, Meulen, V ter, Kat, M & Koprowski, H* Comparison of subacute sclerosing panencephalitis and measles viruses an electron microscope study J virol *7* 176 187, 1971

27 *Raine C S Feldman L A, Sheppard, R D & Bornstein, M B* Ultrastructure of measles virus in cultures of hamster cerebellum J virol *4* 169-181 1969

28 *Raine, C S Feldman L A Sheppard, R D & Bornstein, M B* Ultrastructural study of long term measles infection in cultures of hamster dorsal root ganglion J virol *8* 318-329, 1971

29 *Rapp, F, Gordon, I & Baker, R F* Observations of measles virus infection of cultured human cells. I A study of development and spread of virus antigen by means of immunofluorescence. J biophys. biochem Cytol *7* 43-48, 1960

30 *Rustigian, R* A carrier state in HeLa cells with measles virus (Edmonston strain) apparently associated with non infectious virus. Virology *16* 101-104 1962

31 *Roizman, B & Schluederberg, A E* Virus infection of cells in mitosis II Measles virus infection of mitotic HEp 2 cells. Proc Soc. exp Biol Med (N Y) *106* 320 323 1961

32 *Sever, J L & Zeman W* Measles virus and subacute sclerosing panencephalitis Neurology 18, No 2, 1968

33 *Tawara, T* Fine structure of filaments in dog kidney cell cultures infected with measles virus. Virology *25* 322 1965

34 *Tawara, J T Goodman J R, Imagawa D T & Adams, J M* Fine structure of cellular inclusions in experimental measles Virology *14* 410-416, 1961

35 *Waters, D J Hersh R T & Bussell, R H* Isolation and characterization of measles nucleocapsid from infected cells Virology *48* 278-281, 1972

36 *Waterson, A P* Measles virus Arch ges. Virusforsch *16* 57-80 1965

37 *Waterson A P Cruickshank J G, Laurence G D & Kanarek A D* The nature of measles virus Virology *15* 379 382 1961

38 *Wear, D J, Rabin E R Richardson L S & Rapp, F* Virus replication and ultrastructural changes after induction of encephalitis in mice by measles virus Exp molec Path *9* 405-417 1968

Acta path microbiol scand Section A 81, 3, 291–300, 1973

THE PANCREATIC ISLET CELLS IN INSULAR AMYLOIDOSIS IN HUMAN DIABETIC AND NON-DIABETIC ADULTS

PER WESTERMARK and LARS GRIMELIUS

Department of Pathology, University of Uppsala, Uppsala, Sweden

The relative pancreatic islet cell frequencies were studied in an autopsy material of diabetic and non diabetic 60 year-old and older persons with and without islet amyloidosis The diabetics showed a slightly higher α_1 cell frequency, a higher α_2 cell frequency and a lower β cell frequency than the non diabetics There was a positive correlation between the α_2 cell frequency and the percentage number of islets with amyloid deposits, and a negative correlation between the β cell frequency and this percentage number, the same applied when the effect of diabetes was eliminated by partial correlation analysis The ratio between the nuclear sizes of the β and α_2 cells was fairly constant, even in cases with heavy islet amyloidosis where these sizes were decreased Changes in size of the nuclei were therefore of no importance to the differential counts

Deposition of amyloid in the islets of Langerhans has been said to be accompanied by a reduction in number of the β cells and sometimes of the α cells (*Hartroft* 1950, *Ogilvie* 1964, *Warren et al* 1966) This statement seems to have been based on studies on diabetic individuals only and a quantitative analysis of this problem has apparently not been reported The behaviour of the different endocrine islet cells in islet amyloidosis in non-diabetic persons seems to be almost unknown

The principal aim of this investigation was to study the islet cell frequencies in relation to islet amyloidosis in diabetic and non diabetic adult persons A further aim was to gain more knowledge about the behaviour of the different islet cell types in human maturity onset diabetes In addition, karyometry was performed in an attempt to shed some light on the activity of the α_2 and β cells in islet amyloidosis

Received 14 xi 72 Accepted 15 xii 72

Requests of reprints should be addressed to *Per Westermark*, M D, Department of Pathology, Box 553, S 751 22 Uppsala 1, Sweden.

19*

MATERIAL AND METHODS

The investigation was carried out on a series of autopsy cases. Two groups were studied, one group of cases with maturity onset diabetes and one with no signs of diabetes Only persons 60 years old and older were included in the material All of these had been examined in hospital In the non diabetic group several urine tests for glucose had been performed with negative results and in most cases at least one blood glucose determination with a normal result In one case (case 15), latent diabetes had also been excluded by a glucose tolerance test

A total of 27 autopsy cases, including 11 diabetics, were investigated (see Tables 1 and 2) The mean age of the non diabetics was 76 years and that of the diabetics 73 years The duration of the diabetes varied between 3 and 26 years.

The interval between death and autopsy varied between 3 and 10 hours During this time the bodies were kept at +4° C except for the first two hours The pancreas was removed as quickly as possible at autopsy and pieces from the pancreatic tail, body and head were fixed in 4 per cent for-

maldehyde solution Pieces from the tail were also fixed in Bouin s solution All pieces were dehydrated in a series of graded ethanol, cleared in methyl benzoate and embedded in paraffin Deparaffinized adjacent sections about three-four μ thick from the Bouin fixed specimens were stained with alkaline Congo red (*Puchtler et al* 1962), the Gomori aldehyde fuchsin stain mainly as modified by *Maske* (1955) with ponceau fuchsin as a counterstain (*Hultquist* 1962), the Davenport alcoholic silver nitrate stain as modified by *Hellerstrom and Hellman* (1960) and variant 2 of the Grimelius silver nitrate procedure (*Grimelius* 1968 b)

In every case, the Bouin fixed section stained with alkaline Congo red was studied in a polarization microscope and both the number of islets with amyloid deposits and the total number of islets in the section were determined as described previously (*Westermark* 1972 a) In order to ascertain the representativeness of the Bouin fixed section concerning islet amyloidosis at least five further formalin fixed specimens from different parts of the pancreas were stained with alkaline Congo red and studied in a polarization microscope

Differential counts on Bouin fixed adjacent sections stained with the two silver stains and the Gomori stain were carried out in a binocular microscope with a magnification of X 1250 (immersion lens) and with the aid of a movable stage and a squared grid placed in one of the eyepieces The sections were examined systematically and the islets were differentially counted in the order in which they occurred In the adjacent sections the study was begun in the same area Only cells with nuclei or nuclear fragments in the section were counted The α_1 cells were counted in the Davenport stain the α_2 cells in the Grimelius stain and the β cells in the Gomori stain At least 2000 islet cells were counted in each case and stain (*Hultquist et al* 1948)

Karyometry was performed as described by *Hultquist* (1959) this was done in all cases on 50 α_2 cells in the Grimelius stain with nuclear fast red as a counterstain and on 50 β cells in the Gomori stain The nuclei were traced off at a magnification of approximately X 1500 and the nuclear areas were then determined planimetrically Karyometry of the α_1 cell nuclei was found to be almost impossible with the method used here due to the heavy granulation which often obscured part of the nucleus

In order to clarify the effect of post mortem autolysis on the size of the nucleus pancreatic tissue from a 50 year old non diabetic man was studied The pancreas was removed 5 hours after death One pancreatic specimen was fixed immediately in Bouin s solution The rest of the pancreas was kept at +4° C and further pieces of it were fixed 8 and 10 hours after death Measurements of the nuclei were performed as described above

To obtain a very rough idea of the islet volume in the different cases the proportion of islet area in the total pancreatic area was estimated in the Gomori stained sections by point sampling (*Aherne* 1970) Four hundred hits were counted in each section

RESULTS

The pancreatic tissue was well preserved in all cases and there was no notable post mortem autolysis In most cases there was slight diffuse interacinar and insular fibrosis This was more pronounced in the diabetic than in the non diabetic cases but was not more evident in cases with amyloid deposits than in those without

In the granule stained and silver impregnated sections the different endocrine epithelial cell types could easily be distinguished In islets without amyloid deposits most of the cells were β cells (Fig 1 a) The α cells were concentrated in the periphery and along the islet vessels (Fig 3 a) The few α_1 cells were scattered more randomly within the islets (Fig 2 a) In islets with heavy deposits of amyloid the α_2 cells often predominated (Fig 3 b) The β cells seemed to be less numerous than normally and, if lying close to amyloid deposits, they displayed a wealth of intensely stained granules (Fig 1 b) The border line between the β cells and the amyloid was often indistinct The distribution of the α_1 cells was the same as in normal islets

---

Fig 1 Islets stained with the Gomori aldehyde fuchsin stain

a Islet without amyloid deposits β cells with ordinary granulation are seen × 600

b Islet with heavy amyloid deposits (A) Note the darkly stained β cells adjacent to amyloid × 600

Fig 2 Islets stained with the modified Davenport stain

a A few scattered α_1 cells (arrows) are seen in an islet without amyloid deposits × 370

b Islet with heavy amyloid deposits (A) There are only few α_1 cells (arrows) among the remaining cells × 370

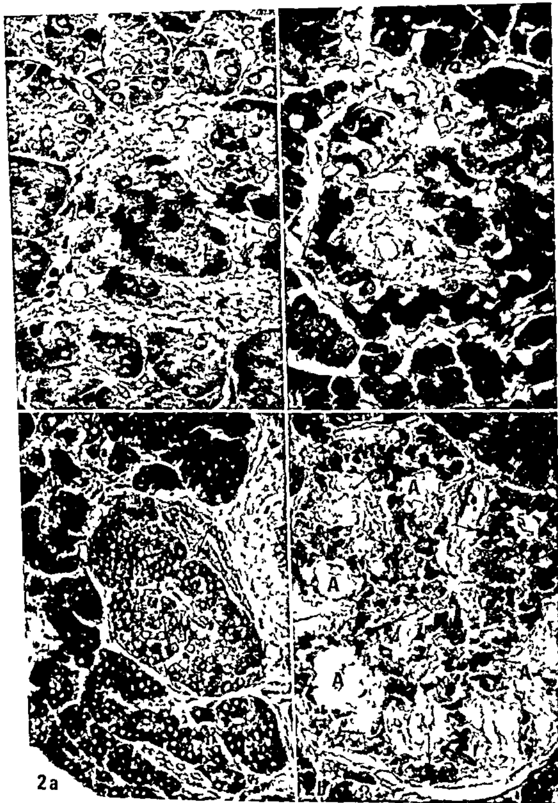

A
A
A
A
A
A
2a

TABLE 1 *Diabetic Persons*

Case No	Sex	Age at death (years)	Duration of diabetes (years)	% islets with amyloid	Differential counts cells in % of total endocrine cells α_1	α_2	β
1	M	69	6	98	11.1	51.9	40.7
2	F	76	13	94	8.2	56.3	37.2
3	F	66	11	33	7.7	64.7	34.0
4	M	77	4	5	9.9	45.0	54.0
5	F	68	4	0	9.7	51.9	47.2
6	F	76	11	95	9.7	58.9	41.7
7	F	71	22	100	10.3	43.2	54.3
8	F	74	26	99	10.0	64.1	35.7
9	F	78	5	87	9.2	64.5	37.4
10	M	65	3	5	13.3	47.8	51.3
11	M	82	16	85	8.9	56.5	39.2

TABLE 2 *Non Diabetic Persons*

Case No	Sex	Age at death (years)	% islets with amyloid	Differential counts cells in % of total endocrine cells α_1	α_2	β
12	F	87	0	12.5	36.7	58.7
13	F	69	0	6.3	30.7	65.6
14	M	84	0	6.4	25.1	66.5
15	M	67	1	7.4	47.5	51.3
16	F	83	32	8.9	40.8	58.2
17	F	84	8	9.1	36.0	61.5
18	F	78	0	7.6	34.0	67.5
19	F	86	0	4.9	36.5	67.1
20	M	71	91	11.5	46.1	50.3
21	M	77	23	6.2	36.1	60.4
22	M	60	8	4.8	35.6	70.7
23	F	67	11	6.8	43.0	56.1
24	M	78	13	13.2	36.8	59.5
25	M	81	14	6.9	55.6	45.5
26	M	69	12	11.6	52.3	45.9
27	F	80	0	8.6	33.6	62.3

TABLE 3 *Coefficients of Correlation in the Simple Correlation Analysis*

	Presence of diabetes	Duration of diabetes (years)	Differential counts α_1	α_2	β
Age	−0.24	0.25	−0.02	−0.25	0.19
Sex	0.13	0.38	0.14	0.07	−0.03
Presence of diabetes	–		0.33	0.72†	−0.74†
Duration of diabetes (years)	–	–	−0.26	0.21	0.25
% islets with amyloid	0.62†	0.63*	0.26	0.62†	−0.68†

*, §, and † denote P<0.05 <0.01 and <0.001, respectively

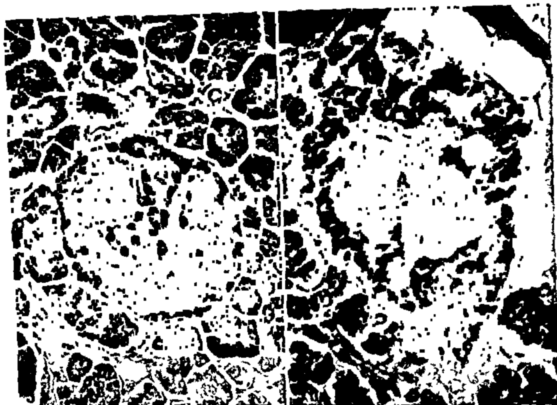


Fig 3 Islets stained with the Grimelius stain

a Islet without amyloid deposits α_2 cells are seen along the vessels and at the periphery of the islet $\times$ 370

b Islet with heavy amyloid deposits (A) Most of the remaining cells are α_2 cells $\times$ 370

(Fig 2 b) Ten (four men and six women) of the 11 diabetics showed amyloid deposits, usually in abundance In the non diabetic group, 10 cases (seven men and three women) exhibited amyloidosis of the islets, being extensive, however, only in one case (case 20) In all cases with islet amyloidosis, deposits were seen in all sections investigated

The quantitative results for the individual persons are given in Tables 1 and 2 Some results of the simple correlation analyses are presented in Table 3 There was a highly significant correlation between the presence of diabetes mellitus and the percentage number of islets with amyloid deposits ($r = 0.62$, $P < 0.001$) There was also a significant correlation between the duration of diabetes and the percentage number of islets with amyloid deposits ($r = 0.63$, $P < 0.05$) In the total material there was no correlation between either sex or age and the percentage number of islets with amyloidosis ($r = 0.10$ and -0.06, respectively)

The proportion of islet tissue in the total pancreatic area in the non-diabetic group was 3 30±0 32 per cent and that in the diabetic group 2.59±0 42 per cent This difference was not significant ($t = 1.35$, $P > 0.10$) On exclusion of the islet amyloid, however the proportion of islet tissue in the non-diabetic group was approximately 3 23±0 32 per cent and that in the diabetic group approximately 1 90±0 29 per cent, this difference was statistically significant ($t = 3.08$, $P < 0.01$) The proportion of islet tissue did not differ significantly between cases with and

TABLE 4 *The Islet Cell Frequencies in Per Cent $\pm$ S E in Non Diabetic Persons with and without Islet Amyloidosis*

	α_1	α_2	β
Persons without islet amyloidosis n = 6	7 7±1 1	32 8±1 8	64 6±1 4
Persons with islet amyloidosis n = 10	8 6±0 9	43 0±2 3	55 9±2 5
t value	0 67	3 50	3 05
Significance	P>0 5	P<0 01	P<0 01

without islet amyloidosis in the non diabetic group, even if the amyloid was excluded (t = 1 43, P>0 10)

Islet Cell Frequencies

The relative islet cell frequencies (in the following called "islet cell frequencies") in the non diabetics without and with islet amyloidosis are given in Table 4 In cases with amyloidosis there was a *significant decrease in* the β cell frequency (t = 3 05, P<0 01) and a significant increase in the α_2 cell frequency (t = 3 50, P<0 01) There was also a small but non significant, increase in the α_1 cell frequency

The islet cell frequencies in the diabetic and non diabetic persons are presented in Table 5 The islet amyloidosis is not taken into consideration here The decrease in the β cell frequency and the *increase in the* α_2 cell frequency in the diabetics are highly significant (t = 5 48 and 5 16, respectively, P<0 001) *There was also a minor increase*

TABLE 5 *The Islet Cell Frequencies in Per Cent $\pm$ S E in Diabetic and Non Diabetic Persons Leaving Islet Amyloidosis out of Consideration*

	α_1	α	β
Non diabetics n = 16	8 3±0 7	32 2±2 0	59 2±1 9
diabetics n = 11	9 8±0 5	55 0±2 3	43 0±2 2
t value	1 90	5 16	5 48
Significance	0 1>P>0 05	P<0 001	P<0 001

TABLE 6 *Coefficients of Partial Correlation in the Multiple Regression Analysis*

Explanatory variables	Dependent variables α_1	α_2	β
Presence of diabetes	0 21	0 54§	−0 55§
% islets with amyloid	0 08	0 33$	−0 42*

* and § denote P<0 05 and <0 01 respectively
$ denotes 0 1>P>0 05

in the α_1 cell frequency in the islets of the diabetics (*t = 1 90, 0 1>P>0 05*)

In the simple correlation analyses (see Table 3) a highly significant correlation was found between the presence of diabetes and the frequency of β and α cells (r = −0 74 and 0 72, respectively, P<0 001) There was a weaker correlation between the presence of diabetes and the frequency of α_1 cells (r = 0 33, 0 1>P>0 05) A highly significant correlation was revealed between the percentage number of islets with amyloid deposits and the frequency of β and of α cells (r = −0 68 and 0 62, respectively P<0 001) There was no significant correlation between the frequency of α_1 cells and the percentage number of islets with amyloid deposits

In the partial correlation analysis (Table 6) there was still a significant correlation between the presence of diabetes and the β and α_2 cell frequencies (r = −0 55 and 0 54 respectively, P<0 01) if the effect of differences in the percentage number of islets with amyloid had been eliminated There was also a significant correlation between the percentage number of islets with amyloidosis and the β cell frequency (r = −0 42 P<0 05) and a somewhat weaker correlation between the former parameter and the α cell frequency (r = 0 33, 0 1>P>0 05) if the effect of diabetes had been eliminated

Karyometry

The effects of the post mortem autolysis are shown in Table 7 It can be seen that there was some shrinkage of the α_2 and β cell

TABLE 7 *Islet Cell Nuclear Sizes (Arbitrary Units) in a 50 Year Old Non Diabetic Man without Islet Amyloidosis at Different Times after Death*

	Hours after death			Maximal decrease of nuclear area in per cent
	5	8	10	
α_2	5 10±0 14	4 74±0 12	4 92±0 16	7 1
β	6 96+0 21	6 50±0 25	6 30±0 31	9 5

TABLE 8 *Nuclear Sizes (Arbitrary Units) of a and β Cells in Non Diabetic Persons with and without Islet Amyloidosis and the Ratio between the β and α_2 Nuclear Sizes*

	α_2	β	β α_2 ratio
Persons without islet amyloidosis $n = 6$	5 48±0 27	7 06±0 26	1 30±0 08
Persons with islet amyloidosis n 10	4 96+0 15	6 87±0 27	1 39±0 05
t value	1 68	0 49	0 95
Significance	P>0 1	P>0 6	P>0 3

TABLE 9 *Nuclear Sizes (Arbitrary Units) ± S E of α_2 and β Cells in Diabetic and Non Diabetic Persons, Leaving Islet Amyloidosis out of Consideration and the Ratio between the β and α_2 Nuclear Sizes*

	α_2	β	β α_2 ratio
Non diabetics n = 16	5 15±0 15	6 94±0 19	1 36±0 04
Diabetics n = 11	4 09±0 15	5 17±0 24	1 28±0 05
(n = 7)	(3 80±0 09)	(4 77±0 25)	(1 26±0 17)
t value	5 03	5 79	1 21
Significance	P<0 001	P<0 001	P>0 2

Values within brackets in the diabetic group refer to persons with heavy islet amyloidosis

nuclei after prolonged autolysis On the other hand, this reduction in size was about the same in the two types of cell, i e between 7 1 and 9 5 per cent of the original nuclear area

The nuclear sizes of the α_2 cells and the β cells in the non diabetic patients with and without amyloidosis are given in Table 8 There was a slight decrease in the size of the nuclei of both these cell types in cases with islet amyloidosis, but the decrease was not significant.

A comparison of the sizes of the nuclei of the α_2 and β cells in the non diabetic and diabetic patients, leaving islet amyloidosis out of consideration, is presented in Table 9 There was a significant decrease in the nuclear size of the α_2 cells ($t = 5\ 03$, $P < 0\ 001$) and of the β cells ($t = 5\ 79$, $P < 0\ 001$) in the diabetic patients The nuclear sizes of the α_2 and β cells in the diabetic patients with heavy amyloidosis are also given in this table These values are seen to be the lowest values observed in this investigation

In all groups presented in Tables 8 and 9 the β cell nuclei were larger than the nuclei of the α_2 cells The ratio between the mean area of the β cell nuclei and the α_2 cell nuclei was, however, relatively constant, varying between 1 26 and 1 39 From this it could be calculated that the ratio between the mean diameter of the β cell and of the α_2 cell nuclei varied between 1 12 and 1 18

DISCUSSION

The correlation between maturity onset diabetes mellitus and islet amyloidosis is well established (cf *Warren et al* 1966) It is also accepted that islet amyloidosis occurs in elderly non-diabetic individuals (*Bell* 1959, *Ehrlich and Ratner* 1961) The significance of the amyloid deposition is, however unknown The islet amyloid in non diabetic persons has been suggested to constitute a sign of prediabetes (*Bell* 1959), but this would seem unlikely since the incidence of islet amyloidosis in elderly patients is very high (*Ludwig and Heitner* 1967, *Schwartz* 1965)

A correlation between the age of the patient and the occurrence of islet amyloidosis has been found by some authors (*Bell* 1952 and 1959, *Ludwig and Heitner* 1967) No

such correlation was obtained in the present series in which the age range was 60 to 87 years In several investigations no relationship between the duration of diabetes and islet amyloidosis has been found (*Bell* 1952, *Le Compte* 1960, *Warren et al* 1966, *Ludwig and Heitner* 1967) The present study, on the other hand, revealed a positive relationship between the degree of amyloidosis and the duration of the diabetes

The heavy granulation of the β cells adjacent to amyloid deposits has been noted previously (*Hartroft* 1950) and has also been demonstrated by electron microscopy (*Westermark* 1972 b) The significance of this finding is unknown

In the literature, the results of differential counts of human pancreatic islets have varied greatly Thus the α cells have been reported to constitute between 6 and 68 per cent of the islet cells in non-diabetic adults (*Creutzfeldt 1953, Creutzfeldt and Theodossiou* 1957, *Ferner* 1942 a and b, *Gepts* 1957 and 1958, *Gomori* 1941, *Hess* 1946, *Hultquist et al* 1948, *Maclean and Ogilvie* 1955, *Seifert* 1954, *Terbruggen* 1948) These variations are probably due to the use of different granule staining methods and methods of silver impregnation and to varying techniques of differential counting Thus a comparison between the cell counts reported in the literature and the present results would seem to be of no value

As seen in Tables 1 and 2 the sum of the percentages of α_1, α_2 and β cells exceeded in most cases one hundred per cent This was probably due to the fact that the α cells were more easily identifiable in the silver stained sections than in the Gomori stained sections and may have been due also to the staining of a few cells both in the Davenport and the Grimelius stain (*Grimelius 1968* a)

The ratio of the number of β cells to α cells has been shown to be lower in diabetic than in non diabetic patients (*Creutzfeldt* 1953, *Creutzfeldt and Theodossiou* 1957 *Ferner* 1942 b, *Gepts* 1957 and 1958, *Gomori* 1941, *Hess* 1946, *Maclean and Ogilvie* 1955, *Seifert* 1954 and 1958, *Terbruggen* 1948) This is in agreement with the findings in the present study The increase of the α_1 and α_2 cell frequencies was probably only relative, since it has been shown that the total mass of α cells is lower in diabetic than in non-diabetic individuals (*Gepts* 1957 and 1958, *Maclean and Ogilvie* 1955) and since, roughly estimated, the proportion of islet tissue in the pancreas, excluding amyloid, was lower in the diabetics than in the non diabetics in the present series. The increase of the α_1 cell frequency in the diabetic group was smaller than that of the α_2 cell frequency and therefore it is not improbable that the total mass of α_1 cells was lower in the diabetic than in the non diabetic persons This contradicts the finding by *Fujita* (1966) who observed an increased number of α_1 cells in diabetic individuals

In most of the previous investigations there has been some overlapping in the islet cell frequencies, with a normal β α ratio in some diabetics and a low β α ratio in a few non-diabetic patients (*Creutzfeldt* 1953, *Creutzfeldt and Theodossiou* 1957, *Hess* 1946 *Maclean and Ogilvie* 1955, *Seifert* 1954, *Terbruggen* 1948) The low values are possibly found in patients with amyloid deposits in the islets In the present series there was no overlapping in the α_2 and β cell frequencies in the non diabetics without islet amyloidosis and the diabetic persons On the other hand some overlapping was observed in the cell frequencies in the non diabetics with and without islet amyloidosis.

The degree of islet amyloidosis in a section is strongly correlated to the percentage number of islets involved and can easily be determined with the aid of the latter (*Westermark* 1972 a) In the present investigation, the mean islet area occupied by amyloid, determined in this way, in the non diabetics with islet deposits was rather small The decrease in the β cell frequency in non diabetic cases with islet amyloidosis might have been due to a greater resistance in the α cells than in the β cells This decrease, however was too advanced to be explained by simple mechanical compression by the small amyloid deposits, at least in the non diabetics This does not, of

course, exclude the possibility that the reduction of the β cell frequency was due to an effect of the amyloid on the β cells Another and perhaps more probable explanation is that the β cell damage is the primary event Electron microscopic studies support this latter proposition (*Westermark* 1972 b) A third possibility is that there was a real increase in the number of α cells

Differences in the size of the nucleus of the islet cells cause systematic overrepresentation of the number of β cells Differential cell counts should thus be combined with determinations of the size of the nucleus in the α and β cells (*Hellman* 1959) In the present investigation, the ratio between the diameters of the β and α_2 cell nuclei in the different groups was fairly constant and the magnitude of this systematic error can therefore be assumed to be constant

As seen in Table 7, post mortem autolysis caused some shrinkage of the nuclei of the different types of islet cells However, this was of about the same magnitude in the α_2 and β cells and is thus of no importance for the differential cell counts

The size of the islet cell nuclei is a function of the activity of the cells (cf *Hultquist* 1965) In the present study, the nuclei of the α_2 and β cells were slightly smaller in non diabetics with amyloidosis than in those without and were significantly reduced in size in diabetic persons, especially in those with massive amyloidosis Thus, in islet amyloidosis there is apparently no increase in the activity of the α_2 or β cells

---

Supported by the *Swedish Medical Research Council* (project No B 73-12 X-102-09 B) the *Research Fund of the Swedish Diabetes Association* and the *Swedish Society for Medical Research* The excellent technical assistance of *Ann Charlotte Hallner*, *Lena Ronning* and *Anders Strand* is greatly appreciated

REFERENCES

Aherne W Quantitative methods in histology J Med Lab Technol 27 160-170 1970

Bell, E T Hyalinization of the islets of Langerhans in diabetes mellitus Diabetes *1* 341-344, 1952

Bell, E T Hyalinization of the islets of Langerhans in non diabetic individuals Amer J Path *35* 801 805, 1959

Creutzfeldt, W Zur Deutung des Silberzellbildes und anderer Pankreasbefunde *beim Diabetes* mellitus und Inseladenom Beitr Path. Anat *113* 133 168, 1953

Creutzfeldt, W & *Theodossiou, A* Die Relation der A und B Zellen in den Pankreasinseln bei Nichtdiabetikern und Diabetikern Beitr Path Anat *117* 235-252, 1957

Ehrlich J C & *Ratner, I M* Amyloidosis of the islets of Langerhans A restudy of islet hyalin in diabetic and nondiabetic individuals Amer J Path *38* 49 59, 1961

Ferner, H Beitrage zur Histobiologie der Langerhansschen Inseln des Menschen mit besonderer Berucksichtigung der Silberzellen und ihrer Beziehung zum *Pankreasdiabetes. Virchows Arch* *309* 87-136, 1942 a

Ferner, H Über morphologisch fassbare Veranderungen der Langerhansschen Inseln beim Pankreasdiabetes Deutsch Z Verd Stoffwech selkrankh *6* 21 29, 1942 b

Fujita, T D Zellen der Pankreasinseln beim Diabetes mellitus mit besonderer Berucksichtigung ihrer argyrophilie Z Zellforsch *69* 363-370, 1966

Gepts, W Contribution à l étude morphologique des ilots de Langerhans au cours du diabete Ann Soc. Roy Sci Med Natur Bruxelles *10* 5-108 1957

Gepts, W *Die histopathologischen Veranderungen* der Langerhansschen Inseln und ihre Bedeutung in der Frage der Pathogenese des menschlichen Diabetes Endokrinologie *36* 185-211, 1958

Gomori, G Observations with differential stains on human islets of Langerhans. Amer J Path *17* 395-406 1941

Grimelius, L A silver nitrate stain for α_2 cells in human pancreatic islets Acta Soc Med Upsal *73* 243-270 1968 a

Grimelius, L The argyrophil reaction in islet cells of adult human pancreas studied with a new silver nitrate procedure Acta Soc Med Upsal *73* 271 294, 1968 b

Hartroft W S The islets of Langerhans in man visualized by phase contrast microscopy Proc Amer Diab Ass. *10* 46-61, 1950

Hellerstrom, C & *Hellman B* Some aspects of silver impregnation of the islets of Langerhans in the rat. Acta Endocr *35* 518-532, 1960

Hellman, B Methodological aspects on the differential cell count of the islet tissue in the rat Acta Path. Microbiol Scand *45* 336-346 1959

Hess W Zur histologischen Diabetesdiagnose an

den Langerhansschen Inseln Schweiz Zeitschr Path Bakt *9* 46–60, 1946

Hultquist, G T Effect of certain sulfhydryl blocking substances on blood sugar and islets of Langerhans in rats. Acta Path Microbiol Scand *47* 153–159, 1959

Hultquist, G T Nuclear size in the cells of Langerhans' islets during starvation in the rat Acta Anat *49* 281–287, 1962

Hultquist, G T An empirical study to ascertain the value of measurement of nuclear size and eccentricity in assessing functional changes in the cells of Langerhans islets Acta Anat. *60* 193–198, 1965

Hultquist, G T, Dahlén, M & Helander, C G Über die Technik bei Darstellung und Zahlung der sogen Silberzellen in den Langerhansschen Inseln Schweiz Zeitschr Path Bakt *11* 570–589, 1948

LeCompte, P M Pathologic anatomy of the pancreas in diabetes mellitus In Diabetes (Williams, R H, ed) New York Paul B Hoeber, Inc. 1960

Ludwig, G & Heitner, H Zur Haufigkeit der Inselamyloidose des Pankreas beim Diabetes mellitus. Zschr Inn Med *22* 814–818, 1967

Maclean, N & Ogilvie, R F Quantitative estimation of the pancreatic islet tissue in diabetic subjects Diabetes *4* 367–376, 1955

Maske, H Eine neue Methode zur Darstellung der A und B Zellen in den Langerhansschen Inseln des Pankreas Klin Wschr *33* 1058 1955

Ogilvie, R F The endocrine pancreas in human diabetes In The structure and metabolism of the pancreatic islets (Brolin, S E, Hellman B Knutson, H, eds), p 499 Oxford Pergamon Press 1964

Puchtler, H, Sweat, F & Levine, M On the binding of Congo red by amyloid J Histochem. Cytochem. *10* 355–364, 1962

Schwartz, P Über Amyloidose des Gehirns, der Langerhansschen Inseln und des Herzens alter Personen Zbl. Allg Path Anat *108* 169–187, 1965

Seifert, G Zur Orthologie und Pathologie des qualitativen Inselzellbildes (nach Bensley – Terbruggen) Virchows Arch *325* 379–396, 1954

Seifert, G Die Diabetestypen und das Inselsystem Deutsch Med Wschnschr *83* 1289–1294 1958

Terbrüggen, A Untersuchungen uber Inselapparat und Inseladenome des Pankreas, insbesondere uber die Zelltypen bei Diabetes mellitus und Spontanhypoglykamie Virchows Arch *315* 407–460, 1948

Warren, S, LeCompte, P M & Legg, M A The

the islets of Langerhans Upsala J Med. Sci *77* 91 94, 1972 a.

Westermark, P Fine structure of islets of Langerhans in insular amyloidosis Virchows Arch. Abt A Path Anat In Press 1972 b

Acta path. microbiol scand Section A. 81, 3, 301–314, 1973

MORPHOMETRIC STUDIES OF THE LEYDIG CELLS IN ELDERLY MEN WITH SPECIAL REFERENCE TO THE HISTOLOGY OF THE PROSTATE

An Analysis in an Autopsy Series

THORSTEIN B HARBITZ

Ullevål Hospital, Department of Pathology, University of Oslo, Oslo, Norway
Head Kristen Arnesen, M D

As part of a comprehensive study of morphological characteristics of the testes, the pituitary gland and the adrenal glands in men with prostatic hyperplasia and neoplasia, Leydig cell weight (W_L) was estimated and analysed in a consecutive autopsy series of 172 men aged 40 years or more W_L varied widely at all ages and decreased gradually with advancing age Protracted illness before death and treatment with steroid hormones other than oestrogens were associated with reduced W_L. In patients with prostatic carcinoma, treatment with oestrogenic hormones apparently caused a rapid disappearance of the Leydig cells Both high and low weights were observed in patients with benign nodular hyperplasia (BNH) alone and in those with carcinoma (C) of the prostate, and no convincing relationship between W_L *per se* and the presence of C or BNH was demonstrable at simple and multiple regression analysis. This implies that an estimation of W_L as such does not discriminate between cases of C and BNH A negative and statistically significant correlation between W_L and age was demonstrated in patients with BNH alone and in those with BNH + C

Clinical observations show that the function and growth of the prostate are closely related to the functional state of the testis Pre pubertal castration prevents the development of benign hyperplasia and carcinoma of the prostate in man (*Moore* 1944), postpubertal castration results in regression of accessory sex glands in experimental animals (*Moore et al* 1930) and man (*Huggins* & *Stevens* 1940) and beneficial effects upon benign hyper plasia (for references see *Wendel et al* 1972) and carcinoma (*Huggins et al* 1941) of the prostate after bilateral orchiectomy have been demonstrated

The interstitial cells of the testis (Leydig cells) constitute the main source of androgens in man (*Hall* 1970) The major role played by androgenic hormones for the function of the prostate has been well documented in experimental animals DNA synthesis and cell proliferation are stimulated (*Coffey et al* 1968 *Fujii* & *Villee* 1969), lipogenesis is accelerated (*Nyden* & *Williams Ashman* 1953, *Farnsworth* & *Brown* 1961) and the synthesis of ribonucleic acids and the incorporation of amino acids into proteins are enhanced (*Hancock et al* 1962, *Kochakian* & *Harrison* 1962, *Liao* & *Williams Ashman* 1962) It has re-

Received 6 xii 72 Accepted 21 xii 72

Requests for reprints should be addressed to *Department of Pathology* Ullevål Hospital, Oslo 1, Norway

cently been shown both in rats (*Anderson & Liao* 1968, *Tveter & Attramadal* 1968) and man (*Hansson et al* 1971) that the prostate has the capacity of selective uptake and accumulation of systemically administered testosterone and other androgenic hormones Hence, a reasonable hypothesis is that changes in the production of or sensitivity to sex hormones in elderly men may be of importance for the development of abnormal growth of the prostate

Only few studies of the relations of the interstitial cells of the testis to the histology of the prostate have been presented In autopsy materials, neither *Teem* (1935) nor *Sommers* (1957) could demonstrate specific changes in the relative number of Leydig cells in patients with either benign hyperplasia or neoplasia as compared to patients with normal histology or diffuse atrophy of the prostate In the subjects with hypertrophied prostates, however, *Teem* observed that the number of Leydig cells gradually diminished with advancing age A retrospective controlled case study by *Koppel et al* (1967) revealed no significant differences in the quantity of Leydig cells in patients with and in patients without prostatic carcinoma

In the present investigation, the relationship between estimates of total weight of the Leydig cell tissue and prostatic hyperplasia and neoplasia has been studied Single variable as well as multiple regression analyses have been performed to correct for the possible influence of cause of death, duration of final illness, steroid hormone treatment, diabetes mellitus, liver cirrhosis, age and body size upon the quantitative morphology of the testis

This investigation of Leydig cell mass forms part of a comprehensive study of the association between pathological growth of the prostate and changes in size and structure of the testes, the pituitary gland and the adrenal glands in elderly men

MATERIAL AND METHODS

The material for the present study was collected from 207 consecutive autopsies of men over 40 years of age The autopsy routine and observations on prostatic histology (*Harbitz & Haugen* 1972), prostatic weight (*Haugen & Harbitz* 1972) and testis weight (*Harbitz* 1973) in the same patients were reported previously

One patient with seminoma of the testis and four patients with secondary neoplastic invasion of the testes or prostate were excluded Those who had undergone prostatic surgery (24), treatment with oestrogenic hormone (diethylstilboestrol) (4) or both (2) were analysed separately Thus, 172 patients were included in the main analysis of Leydig cell weight

Clinical data were recorded from the clinical notes and prepared for computer analysis

Prostate

[illegible]

ration (AGP) diffuse atrophy (DA) or [illegible] histology (N) was recorded from the study of 4-6 [illegible]

The histological findings in the prostates of the 172 cases included in the main analysis are presented in Table 1

TABLE 1 *Histology of the Prostate by Age in 172 Patients*

Age	N	DA	BNH	C+BNH	C	AGP+BNH	AGP
40–49	1	1	2	0	0	0	0
50–59	11	5	10	3	1	5	0
60–69	7	1	22	15	5	6	1
70–79	0	1	28	21	0	5	1
80 +	0	0	9	9	0	2	0
Total	19	8	71	48	6	18	2

N = normal histology, DA = diffuse atrophy BNH – benign nodular hyperplasia C – carcinoma AGP = atypical glandular proliferation

Testis

After the unfixed testis had been weighed, the two polar cupolas were cut off and the specimen immerged in Helly's fluid. After two hours, a deep incision parallel and just cranial to the equator of the testis was made to ensure adequate penetration of the fixative.

After fixation for 24 hours, three transverse slices for processing and paraffin embedding were cut through the equatorial part and midportions of the cranial and caudal halves respectively. One total section from each block was cut with the microtome set at 2 µm, mounted with the rete margin oriented towards the length of the rectangular slide, and stained with phosphotungstic acid haematoxylin (PTAH).

Histometric examination. Leydig cell mass was assessed by point sampling at 10x objective magnification, using an ocular square-ruled network with 81 fixed intersection points.

In general, the sections were approximately circular, and point sampling was performed in four visual fields along the largest section diameter parallel to the slide length, positioned so as to be representative of four concentric areas of approximately equal size (Fig. 1). The diameter (d) was measured directly with the microscope crossboard scale, whereafter the distances from the centre of each point sampling area to the middle of the diameter were calculated as $\frac{1}{2}\left(\frac{d}{4}\right)$, $\frac{1}{2}\left(\frac{d}{4}+\frac{d}{4}\sqrt{2}\right)$, $\frac{1}{2}\left(\frac{d}{4}\sqrt{2}+\frac{d}{4}\sqrt{3}\right)$ and $\frac{1}{2}\left(\frac{d}{4}\sqrt{3}+\frac{d}{2}\right)$, respectively (*Stalsberg*, personal communication). This procedure and point sampling were repeated for one section from each of the three blocks from each testis.

Thus a total of 972 points were counted in each testis*. From the number of hits on Leydig cells (n_L) and the correspondent testis weight (W_t), the total weight of Leydig cells (W_L) was estimated as $\frac{n_L}{972} \times W_t$. Possible differences in specific weights of individual tissue components were disregarded. The sum of W_L for the right and the left testis was taken as the Leydig cell weight in each case.

* In two testes weighing less than 5 grams each, only the sections from the upper and lower halves were counted. Only the equatorial sections were counted in a case in which the testes weighed approximately 1 gram each. If the sections were so small that neighbouring network projections overlapped (24 sections from 11 cases), point sampling was performed in a reduced number (3) of visual fields. In such instances the number of hits on Leydig cells was always transformed to hits per 972 points.

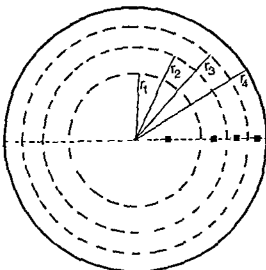


Fig. 1. Schematic drawing of a total section of the testis to demonstrate the location of the visual fields (squares) used for histometry. The visual fields, located along the diameter (d), are representative for concentric areas of equal size: $r_1 = \frac{d}{4}$, $r_2 = \frac{d}{4}\sqrt{2}$, $r_3 = \frac{d}{4}\sqrt{3}$, $r_4 = \frac{d}{2}$.

Statistical Analysis

Modified Student's t tests accounting for unequal variances and numbers of individuals were used for testing differences between arithmetic means and for testing differences between slopes of regression lines (*Snedecor & Cochran* 1967), n_A and n_B being the number of observations in the groups to be compared, p-values were based on the least of $n_A - 1$ and $n_B - 1$ (for means) and $n_A - 2$ and $n_B - 2$ (for slopes) degrees of freedom. P values below 0.05 were regarded statistically significant.

Adjustment for age differences was performed according to the indirect method of standardization (*Armitage* 1971) using the age-specific mean Leydig cell weights of the main material of 172 patients as standard weights.

Multiple regression analysis. Stepwise and full multiple regression analysis were applied as previously described (*Haugen & Harbitz* 1972, *Harbitz* 1973) using Leydig cell weight (X_1) as the *dependent* variable. The following factors, either binariate (1, 0, labelled X_2 to X_{12}) or continuous (labelled X_{13} to X_{15}), were treated as *explanatory* (independent) variables.

Histology of the Prostate

X_2 Benign nodular hyperplasia (BNH)

X_3 Atypical glandular proliferation (AGP)

X_4 Carcinoma (C)
X_5 Diffuse atrophy (DA)

Cause of Death

X_6 Cardiovascular disease§
X_7 Malignant tumour

Duration of Final Illness

X_8 1–7 days
X_9 > 7 days

Other

X_{10} Steroid hormone treatment†
X_{11} Diabetes mellitus
X_{12} Liver cirrhosis
X_{13} Age
X_{14} Body weight
X_{15} Body length

In case of bivariate variables, the presence of the characteristic in question gives the variable the value 1, whereas the absence of this characteristic gives the variable the value 0

A linear regression of X_1 on X_{2-15} was assumed, and the multiple regression equation

$$X_1 = b_0 + b_2X_2 + b_3X_3 + \quad\quad + b_{15}X_{15}$$

was employed, b_0 being a constant and b_n the regression coefficient for X_1 on X_n

Differences between regression coefficients for the various groups of prostatic histology as calculated at the full regression analysis, were tested by an F test (*Scheffé* 1959)

The analysis was based on a standard programme for multiple regression (NRSR) developed at The Norwegian Computing Center, Oslo and was conducted on a Univac 1108 computer

Reproducibility The reproducibility of the method for determining Leydig cell weight was tested by duplicate estimations in 11 randomly selected cases The sections from both testes in these cases were recounted blindly, whereafter the pairs of weights were compared (Fig 2) The standard deviation of a single weight estimate from a given set of sections i e the method error was computed from the usual formula $\sqrt{\Sigma D^2/2n}$, where D denotes the difference between duplicates and n the number of pairs of weights compared The result was 0 37 The number of duplicate measurements was small, and the high method error (39 4 per cent of the total mean Leydig cell weight) can largely be ascribed to the large absolute deviation in one case with very high weight. Nevertheless it is apparent from Fig 2 that the error of the method is considerable even at low weights.

§ Includes death from myocardial infarction (49 cases), cerebrovascular and peripheral vascular disease (16 + 8 cases) rheumatic valvular disease (4 cases), miscellaneous cardiovascular disorders (11 cases)

† Other than oestrogenic hormones Includes treatment with corticosteroids (7 cases), anabolic steroids (nortestosterone) (5 cases), or both (11 cases)

RESULTS

The frequency distribution of Leydig cell weights in the main series of 172 patients is presented in Fig 3, which demonstrates a positive skewness The weights ranged from zero to 5 21 grams, with a median of 0 76 grams

The variation in Leydig cell weights was seen at all ages as shown in Fig 4 a and as expressed by the high standard deviations within 10 year age groups in Table 2 A gradual decrease in mean weights was observed after the age of 50 years The negative correlation between Leydig cell weight and age in men over 40 years of age was statistically significant ($p < 0\ 01$), but the correlation coefficient was low ($r = -0\ 229$)

The frequency of Leydig cell weights below 0 75 grams increased with advancing age from 37 per cent (13/35) among men 50–59 years to 80 per cent (16/20) among men over 80 years Among patients with Leydig cell weights below 0 25 grams practically all (27/29) had protracted illness (lasting more than 7 days) before death, and 65 per cent (19/29) died from a non prostatic malignancy Weights higher than 1 25 grams were observed in 15 of 35 men in the sixth decade and in none of those over 80 years of age

Table 3 presents the mean Leydig cell weight in relation to cause of death, duration of final illness steroid hormone treatment, diabetes mellitus and liver cirrhosis In patients who died from malignant tumours mean Leydig cell weight was significantly lower than in those dying from cardiovascular diseases ($p < 0\ 001$) or other causes ($p < 0\ 05$) A gradual decrease in Leydig cell weight was observed when duration of the final illness was prolonged In patients whose final illness had lasted for only 1–7 days a marked and statistically significant reduction ($p < 0\ 02$) of Leydig cell weight was demonstrable as compared with those dying within 1 day after onset of symptoms A further decrease was

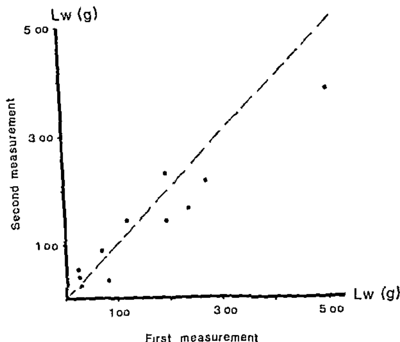


Fig 2 Reproducibility for Leydig cell weight (Lw) estimation in 11 cases examined two times

seen in cases of final illness of more than 7 days duration Patients who had received steroid hormones other than oestrogens and those with liver cirrhosis had low Leydig cell weights, whereas the mean weight in diabetics was higher than the total mean

Within the groups of different histological diagnoses of the prostate, patients with both atypical glandular proliferation and benign hyperplasia (AGP + BNH) had the highest mean Leydig cell weight (Table 4) However the standard deviations were high in all groups The mean weights in patients with benign hyperplasia either alone (BNH) or in combination with carcinoma of the prostate (C + BNH) were nearly identical, and similar to that in patients with normal prostatic histology The decrease in Leydig cell weight with age was statistically significant at correlation analysis both in patients with benign nodular hyperplasia alone ($r = -0.413$, $p < 0.001$) and in patients with both benign nodular hyperplasia and carcinoma of the prostate ($r = -0.343$, $p < 0.05$)

Fig 4 presents scatter diagrams of Leydig cell weight by age for the whole main series of 172 cases and within groups with different prostatic histology Patients with normal histology showed a wide scatter of Leydig cell weights, and both high and low weights occurred (Fig 4 b) Diffuse atrophy of the prostate was associated with low weights at

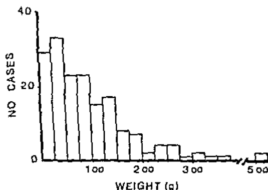


Fig 3 Distribution of Leydig cell weight at autopsy in the main series of 172 men aged 40 years or more

TABLE 2 *Leydig Cell Weight (Grams) in Men Previously not Subjected to Prostatic Surgery or Treatment with Oestrogenic Hormones*

Age	No patients	Leydig cell weight		
		Mean	S D	Range
40–49	4	0 94	0 68	0 21–1 64
50 59	35	1 24*	1 11	0 00–5 00
60 69	57	1 10*	0 95	0 00–5 21
70–79	56	0 84§	0 70	0 12–3 15
80 +	20	0 50	0 27	0 10–0 96
All	172	0 94	0 82	0 00 5 21

§ and * Differing from that in men 80 years or more at the 1 and 0 1 per cent level of significance
S D Standard deviation

all ages (Fig 4 c) Leydig cell weights in the groups of patients with atypical glandular proliferation, benign hyperplasia alone, and carcinoma of the prostate with or without benign hyperplasia, all varied widely, although the variation decreased with advancing age (Fig 4 d–f) Weights below 0 25 grams were more frequent among men carrying prostatic carcinoma (14/54) than in those with benign nodular hyperplasia only (8/71) In patients with prostatic carcinoma, Leydig cell weights showed the same variation whether or not benign hyperplasia was present

To compare the association between Leydig cell weight and age in various groups of prostatic histology, individual regression lines are presented in Fig 5 Only patients aged more than 50 years were considered, since carcinoma and atypical glandular proliferation were not observed before this age The coefficient of the linear regression for men with benign nodular hyperplasia only (BNH) ($b = -0\ 026$) did not differ significantly from that ($b = -0\ 044$) in patients with both prostatic carcinoma and benign hyperplasia (C + BNH) ($p > 0\ 30$) The regression line for patients with atypical glandular proliferation accompanied by benign hyperplasia ($b = -0\ 035$) was nearly parallel to that for the BNH group For reasons of comparison the regression line for subjects with normal prostatic histology, who were all under 70 years of age, is also presented A positive cor

TABLE 3 *Leydig Cell Weight (Grams) and Cause of Death Duration of Final Illness Steroid Hormone Treatment, Diabetes Mellitus and Liver Cirrhosis*

	No patients	Leydig cell weight		
		Observed mean	S D	Age adjusted mean
Cause of death				
Cardiovascular disease	88	1 14	0 85	1 13
Malignant tumour	44	0 59	0 79	0 55
Other causes	40	1 01	0 91	1 00
	172			
Duration of final illness				
< 1 day	37	1 62	1 10	1 49
1 7 days	35	1 06	0 62	0 98
> 7 days	100	0 70	0 72	0 71
	172			
Other				
Steroid hormone treatment*	23	0 35	0 27	0 33
Diabetes mellitus	8	1 08	0 98	1 28
Liver cirrhosis	5	0 63	0 86	0 54
All	172	0 94	0 82	

S D Standard deviation
* Other than oestrogenic hormones

TABLE 4 *Leydig Cell Weight (Grams) by Histology of the Prostate* and Age*

Age	N	DA	BNH	C+BNH	C	AGP+BNH	AGP
40–49	(0.21)	(0.53)	1.50	–	–	–	–
50–59	1.16	0.38	1.08	2.30	(2.52)	1.72	–
60–69	1.31	(0.37)	1.16	0.99	1.00	1.31	(0.11)
70–79	–	(0.42)	0.71	0.83	–	1.64	(1.08)
80 +	–	–	0.47	0.46	–	0.80	–
All	1.16	0.41	0.89	0.90	1.26	1.46	0.60
S D	0.86	0.32	0.57	1.13	0.74	1.11	0.69

* For abbreviations and number of cases, see Table 1
Figures in brackets refer to single observations
S D Standard deviation

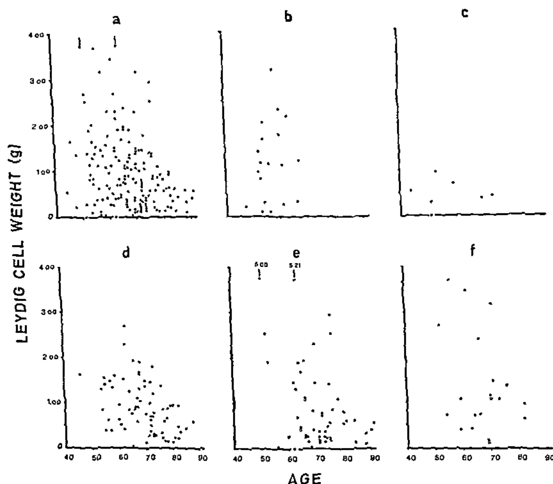


Fig 4 Leydig cell weight plotted against age in the main series of 172 men over 40 years of age (a), and in subgroups of cases with normal histology (b), diffuse atrophy (c), benign nodular hyperplasia only (d), carcinoma with (●) or without (O) benign nodular hyperplasia (e), and atypical glandular proliferation with (●) or without (O) benign nodular hyperplasia (f) of the prostate

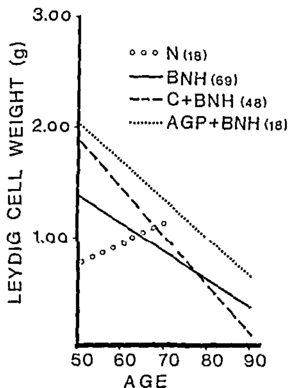


Fig 5 Regression of Leydig cell weight on age in men more than 50 years of age with benign nodular hyperplasia only (BNH), benign nodular hyperplasia and carcinoma (BNH + C), benign nodular hyperplasia and atypical glandular proliferation (BNH + AGP), and normal histology (N) of the prostate Figures in brackets indicate number of cases in each group

relation between Leydig cell weight and age in this group of patients might be assumed from the slope of the regression line (b = 0 018), but the correlation coefficient was far from significant (r = 0 103)

The age specific mean Leydig cell weights in 24 patients who had previously undergone prostatic surgery for benign hyperplasia did not differ significantly from those in non-operated cases (Table 5)

Among the six patients who received diethylstilboestrol for prostatic carcinoma, four had Leydig cell weights at zero, all of whom had been treated for more than 8 months before death (Table 6) Only two of the 172 cases who had not received oestrogen had Leydig cell weights at zero (Fig 4 a) Two patients who had oestrogen treatment for one month only had weights of 0 25 and 1 25 grams

Prior to the multivariate analysis, simple correlation analysis in the main material of 172 patients was performed as a preparatory procedure, and the relationship (expressed as correlation coefficients) between Leydig cell weight and the individual explanatory variables to be included in the multiple regression (X_{2-15}) appears from Table 7 Leydig cell weight was positively correlated at the 5 per cent level of significance with cardiovascular disease as a cause of death and with body weight, but the correlation coefficients were low A negative correlation was observed between Leydig cell weight and malignant tumour as a cause of death, long duration of final illness, steroid hormone treatment and age Again the coefficients of correlation were

TABLE 5 *Leydig Cell Weight (Grams) by Age in Patients Previously Subjected to Prostatic Surgery for Benign Nodular Hyperplasia*

Age	No patients	Leydig cell weight	
		Mean	S D
50–59	3	0 60	0 48
60–69	5	1 13	0 81
70–79	7	0.99	0 35
80 +	9	0 69	0 40
All	24	0 86	0 51

S D : Standard deviation

TABLE 6 *Leydig Cell and Testis Weights (Grams), Age (Years) and Duration of Treatment in Patients who Received Diethylstilboestrol*

Age	Duration of oestrogen treatment	Leydig cell weight	Testis weight*
69	1 month	1 25	15 5
70	1 "	0 25	18 6
68§	8 "	0 00	22 2
66§	12 "	0 00	1 9
76	4 years	0 00	14 9
79	4 "	0 00	10 7

* For details, see *Harbitz* (1973)
§ Previously subjected to transvesical prostatectomy

TABLE 7 *Relationship between Leydig Cell Weight and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variable	X_1 Leydig cell weight (n_1 = 172)	
	Correlation coefficient	Significant at level
Histology of the prostate§		
X_2 BNH (n = 137)	–0 018	0 810
X_3 AGP (n = 20)	0 190	0 013
X_4 C (n = 54)	–0 073	0 340
X_5 DA (n = 8)	–0 146	0 056
Cause of death		
X_6 Cardiovascular disease (n = 88)	0 261	0 001
X Malignant neoplasm (n = 44)	–0 258	0 001
Duration of final illness		
X_8 1–7 days (n = 35)	0 072	0 345
X_9 > 7 days (n = 100)	–0 355	< 0 001
Other		
X_{10} Steroid hormone treatment* (n = 23)	–0 287	< 0 001
X_{11} Diabetes mellitus (n = 8)	0 037	0 629
X_{12} Liver cirrhosis (n = 5)	–0 066	0 391
X_{13} Age (n = 172)	–0 229	0 003
X_{14} Body weight (n = 172)	0 282	< 0 001
X_{15} Body length (n = 172)	0 051	0 506

§ For abbreviations, see Table 1

n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continuous variables)

n_1 Number of cases in which Leydig cell weight was recorded

* Other than oestrogenic hormones

low, only that for protracted illness (X_9) being over 0 35 A statistically significant, but low correlation between Leydig cell weight and the presence of atypical glandular proliferation of the prostate was demonstrable, whereas the correlations to the other histological groups were not statistically significant

Multiple Regression Analysis

In the stepwise procedure of multiple regression analysis long duration of final illness (X_9) caused the greatest reduction in the variance of Leydig cell weight, and was selected before steroid hormone treatment (X_{10}), age (X_{13}), 1–7 days' duration of final illness (X_8), and atypical glandular proliferation of the prostate (X_3) The multiple correlation coefficient (R) for these five regressors was rather low (0 500) If all groups of prostatic histology were included in the multiple regression, the multiple correlation coefficient increased only to 0 510 (Table 8) The partial correlation and regression coefficients, as well as their levels of significance, for the relationship between Leydig cell weight and duration of final illness (X_{8-9}), steroid hormone treatment (X_{10}) and age remained practically unchanged Thus, the explanatory value of the regression (R^2) increased only from 25 to 26 per cent by the inclusion of the groups of prostatic histology other than AGP The partial regression coefficient was at a statistically significant level for AGP (X_3), but not for any of the other groups of prostatic histology The partial regression coefficients for the groups of prostatic histology did not differ individually at the chosen level of significance ($F = 1\ 768$, $f_1 = 4$, $f_2 = 163$, $p > 0\ 10$)

TABLE 8 *Leydig Cell Weight and Histology of the Prostate Full Regression Analysis*

Explanatory variable	X_1 *Leydig cell weight* (n_1 = 172) Partial correlation coefficient	Partial regression coefficient	Significant at level
Histology of the prostate§			
X_2 BNH (n = 137)	0 034	−0 073	0 667
X_3 AGP (n = 20)	0 170	0 398	0 029
X_4 C (n = 54)	0 049	0 080	0 536
X_6 DA (n = 8)	0 099	−0 387	0 206
Other			
X_9 > 7 days (n = 100)	−0 305	−0 608	< 0 001
X_{13} Age (n = 172)	−0 238	−0 018	0 009
X_{10} Steroid hormone treatment* (n = 23)	−0 200	−0 453	0 010
X_8 1–7 days (n = 35)	−0 184	−0 411	0 018
Multiple correlation coefficient (R)	0 510		< 0 001

§ For abbreviations, see Table 1
n and n_1 For explanation, see Table 7
* Other than oestrogenic hormones.

COMMENT

The microscopical recognition of Leydig cells was clear, although some variation in their appearance was present Singular cells might be seen, but aggregates of Leydig cells were most common, confirming previous observations by *Teem* (1935) Clusters of Leydig cells frequently adopted the shape of the vacant space between adjacent seminiferous tubules and vessels Cytological changes due to post mortal autolysis were sometimes prominent, and a distinction between various types of adult Leydig cells (*Sniffen* 1950, *Mancini et al* 1963, *Payne et al* 1971) was not attempted

Previous reports on Leydig cell quantity in the human testis at various ages are conflicting *Stieve* (1930), *Sniffen* (1950) and *Sokal* (1964) held that no change in the number of Leydig cells occurs in advanced age, whereas *Teem* (1935) and *Sargent & McDonald* (1948) reported diminishing quantities of Leydig cells with increasing age In these studies, however only relative estimates have been given, and such measures do not necessarily reflect the true quantity of Leydig cells The size of the testis must be taken into account to know the total Leydig cell quantity

Testosterone is the most potent among natural androgens in man, and the greater part of this hormone is produced in the interstitial cells of the testis (*Hall* 1970) The total mass of these cells may be considered to relate to androgen synthesis a correlation between androgen production and the absolute number of Leydig cells has been demonstrated (*Tillinger et al* 1955) The observed decrease in Leydig cell weight with advancing age coincides with previous observations of lowered testosterone production rates (*Kent & Acone* 1966, *Isurugi* 1967) and declining plasma testosterone levels (*Vermeulen et al* 1972) in old men

In accordance with previous reports on relative estimates of Leydig cell amounts (*Stieve* 1930 *Teem* 1935, *Sargent & McDonald* 1948, *Sniffen* 1950, *Roosen Runge* 1956) both total and age specific Leydig cell weights varied over a wide range The variation coefficients ranged from 54 to 89 per cent, which may agree with the wide variation (variation coefficients 34 68 per cent) observed in plasma testosterone levels in adult men at all ages (*Vermeulen et al* 1972) A

number of observations of low Leydig cell weights were linked to long duration of the terminal illness

In the present series, simple correlation analysis suggested a negative association be tween Leydig cell weight and death from malignant neoplasms and protracted illness which are obviously interrelated At multiple regression analysis only the correlation to more than 7 days' duration of terminal illness was confirmed This probably implies that the effect on Leydig cell weight is primarily from long duration of the final illness These ob servations are in accord with recent reports on lowered plasma testosterone levels in patients with advanced malignant conditions and other chronic disease (*Young & Kent* 1968, *Robinson & Thomas* 1971)

Administration of steroid hormones other than oestrogens was apparently accompanied by a reduction in Leydig cell weight Nearly all patients who received this treatment died from a malignant neoplasm after long duration of the terminal illness To a certain extent therefore, the lowered Leydig cell weight in this group of patients only reflects an inter relation between steroid hormone treatment, protracted disease and death from malignant neoplasm On the other hand, the results of the multiple regression analysis suggested that Leydig cell weight was negatively correlated to the treatment with steroid hormones *per se* These hormones were, in the majority of cases anabolic steroids, which are derivatives of testosterone Administration of low doses of androgens in experimental animals (*Selye & Albert* 1942, *Ludwig* 1950) and testosterone administration to men (*Heller et al* 1950) produces marked atrophy or disappearance of Leydig cells, probably as a result of a suppressing effect on the gonadotropic function of the pituitary gland Although the andro genic activity of anabolic steroids is consid ered to be relatively weak, gonadotropic sup pression is the most likely mechanism leading to reduced quantity of Leydig cells in men using these hormones.

In experimental diabetes in animals, a marked reduction in the number of Leydig cells has been observed (*Schöffling et al* 1967) In the present series, no association between the amount of Leydig cells and diabetes mellitus was observed, confirming the observations by *Federlin et al* (1965) on the relative number of Leydig cells in young men with diabetes mellitus

Liver cirrhosis in men is accompanied by an excess of circulating oestrogens (*Glass et al* 1940, *Korenman et al* 1969) and low plasma levels of testosterone (*Coppage et al* 1965) The few cases with liver cirrhosis included in the present series does not permit any conclusions as to the Leydig cell quantity or their functional capacity in such patients The low mean Leydig cell weight may well be attributed to interrelations between liver cirrhosis and other depressing factors in this series

A convincing relationship between Leydig cell weight *per se* and carcinoma or benign hyperplasia of the prostate was not demon strable at either single variable nor multiple regression analysis, and the individual partial regression coefficients did not differ significantly This means that an estimation of Leydig cell quantity does not discriminate be tween cases with benign hyperplasia and carcinoma of the prostate

The highest mean Leydig cell weights were observed in cases with atypical glandular pro liferation accompanied by benign hyperplasia of the prostate At multiple regression ana lysis, atypical glandular proliferation was the only histological pattern which revealed a correlation to the Leydig cell weight at the chosen level of significance However, the significance level for the partial correlation between atypical glandular proliferation *per se* and Leydig cell weight was low and may be due to chance only

Leydig cell weight in patients who had undergone prostatic surgery did not differ from that in non-operated cases This indicates that the synthesis of androgens and their influence upon remaining glandular tissue is unaltered after removal of hyperplastic prostatic tissue

Oestrogens are potent inhibitors of the re-

TABLE 8 *Leydig Cell Weight and Histology of the Prostate Full Regression Analysis*

Explanatory variable	Y_1 Leydig cell weight (n_1 = 172) Partial correlation coefficient	Partial regression coefficient	Significant at level
Histology of the prostate§			
X_2 BNH (n = 137)	−0 034	−0 073	0 667
X_3 AGP (n = 20)	0 170	0 398	0 029
X_4 C (n = 54)	0 049	0 080	0 536
X_6 DA (n = 8)	−0 099	−0 387	0 206
Other			
X_9 > 7 days (n = 100)	−0 305	−0 608	< 0 001
X_{13} Age (n = 172)	0 238	−0 018	0 009
X_{10} Steroid hormone treatment* (n = 23)	−0 200	−0 453	0 010
X_8 1–7 days (n = 35)	−0 184	−0 411	0 018
Multiple correlation coefficient (R)	0 510		< 0 001

§ For abbreviations, see Table 1
n and n_1 For explanation, see Table 7
* Other than oestrogenic hormones

COMMENT

The microscopical recognition of Leydig cells was clear, although some variation in their appearance was present Singular cells might be seen, but aggregates of Leydig cells were most common, confirming previous observations by *Teem* (1935) Clusters of Leydig cells frequently adopted the shape of the vacant space between adjacent seminiferous tubules and vessels Cytological changes due to postmortal autolysis were sometimes prominent, and a distinction between various types of adult Leydig cells (*Sniffen* 1950, *Mancini et al* 1963, *Payne et al* 1971) was not attempted

Previous reports on Leydig cell quantity in the human testis at various ages are conflicting *Stieve* (1930), *Sniffen* (1950) and *Sokal* (1964) held that no change in the number of Leydig cells occurs in advanced age, whereas *Teem* (1935) and *Sargent & McDonald* (1948) reported diminishing quantities of Leydig cells with increasing age In these studies, however, only relative estimates have been given, and such measures do not necessarily reflect the true quantity of Leydig cells The size of the testis must be taken into account to know the total Leydig cell quantity

Testosterone is the most potent among natural androgens in man, and the greater part of this hormone is produced in the interstitial cells of the testis (*Hall 1970*) The total mass of these cells may be considered to relate to androgen synthesis a correlation between androgen production and the absolute number of Leydig cells has been demonstrated (*Tillinger et al* 1955) The observed decrease in Leydig cell weight with advancing age coincides with previous observations of lowered testosterone production rates (*Kent & Acone* 1966, *Isurugi* 1967) and declining plasma testosterone levels (*Vermeulen et al* 1972) in old men

In accordance with previous reports on relative estimates of Leydig cell amounts (*Stieve* 1930, *Teem* 1935, *Sargent & McDonald* 1948, *Sniffen* 1950 *Roosen Runge* 1956), both total and age specific Leydig cell weights varied over a wide range The variation coefficients ranged from 54 to 89 per cent, which may agree with the wide variation (variation coefficients 34–68 per cent) observed in plasma testosterone levels in adult men at all ages (*Vermeulen et al 1972*) A

15 *Haugen, O A* & *Harbitz, T B* Prostatic weight in elderly men An analysis in an autopsy series Acta path microbiol scand Sect A *80* 769–777, 1972

16 *Heller, C G, Nelson, W O, Hill, I B, Henderson, E, Maddock, W O, Jungck, E C, Paulsen, C A* & *Mortimore, G E* Improvement in spermatogenesis following depression of the human testis with testosterone Fertility & Sterility *1* 415–420, 1950

17 *Huggins, C* & *Stevens, R A* The effect of castration on benign hypertrophy of the prostate in man J Urol *43* 705–714, 1940

18 *Huggins, C, Stevens, R E, Jr* & *Hodges, C V* Studies on prostatic cancer II The effects of castration on advanced carcinoma of the prostate gland Arch Surg *43* 209–223, 1941

19 *Isurugi, K* Plasma testosterone production rates in patients with prostatic cancer and benign prostatic hypertrophy J Urol *97* 903 908, 1967

20 *Kent, J R* & *Acone, A B* Plasma testosterone levels and aging in males In Vermeulen A & Exley, D (Eds) Androgens in Normal and Pathological Conditions. Excerpta Medica Foundation, Internat. Congr Series *101* 31–35 1966

21 *Kochakian, C D* & *Harrison, D G* Regulation of nucleic acid synthesis by androgens Endocrinology *70* 99–108, 1962

22 *Koppel, M, Meranze, D R* & *Shimkin, M B* Characteristics of patients with prostatic carcinoma A control case study on 83 autopsy pairs J Urol 98 229 233, 1967

23 *Korenmann, S G, Perrin, L E* & *McCallum, T* Estradiol in human plasma Demonstration of elevated levels in gynecomastia and in cirrhosis (Abstr no 144) J clin Invest *48* 45a 1969

24 *Liao, S* & *Williams Ashman, H G* An effect of testosterone on amino acid incorporation by prostatic ribonucleoprotein particles Proc. Nat Acad Sci. *48* 1956–1964 1962

25 *Ludwig, D J* *The effect of* androgen on spermatogenesis Endocrinology *46* 453–481, 1950

26 *Mancini R E Vilar, O, Lavieri, J C, Andrada, J A* & *Heinrich, J J* Development of Leydig cells in the normal human testis A cytological, cytochemical and quantitative study Amer J Anat *112* 203–214, 1963

27 *Moore, C R, Price, D* & *Gallagher, T F* Rat prostate cytology as a testis-hormone indicator and the prevention of castration changes by testis-extract injections. Amer J Anat *45* 71–107 1930

28 *Moore R A* Benign hypertrophy and carcinoma of the prostate. Occurrence and experimental production in animals. Surgery *16* 152 167, 1944

29 *Nyden, S J* & *Williams Ashman, H G* Influence of androgens on synthetic reactions in ventral prostate tissue Amer J Physiol *172* 588–600, 1953

30 *Payne, A H, Jaffe, R B* & *Abell, M R* Gonadal steroid sulfates and sulfatase III Correlation of human testicular sulfatase, 3β hydroxysteroid dehydrogenase isomerase, histologic structure and serum testosterone J Clin Endocr 33 582–591, 1971

31 *Robinson, M R G* & *Thomas, B S* Effect of hormonal therapy on plasma testosterone levels in prostatic carcinoma. Brit med J *4* 391–394, 1971

32 *Roosen Runge, E C* Quantitative investigations on human testicular biopsies I Normal testis Fertility & Sterility *7* 251–261, 1956

33 *Sargent, J W* & *McDonald, J R* A method for the quantitative estimate of Leydig cells in the human testis Proc. Mayo Clin *23* 249–254, 1948

34 *Scheffe, H* The analysis of variance John Wiley & Sons, Inc, New York 1959 p 25 51

35 *Schoffling K, Federlin, K, Schmitt, W* & *Pfeiffer, E F* Histometric investigation on the testicular tissue of rats with alloxan diabetes and Chinese hamsters with spontaneous diabetes Acta endocr *54* 335–346, 1967

36 *Selye, H* & *Albert S* The effect of various steroids in intact male rats Amer J Med Sci *204* 876–884, 1942

37 *Snedecor, G W* & *Cochran, W G* Statistical methods 6 ed The Iowa State University Press, Ames Iowa 1967 593 pp

38 *Sniffen, R C* The testis I The normal testis. Arch Path *50* 259 284, 1950

39 *Sohal, Z* Morphology of the human testes in various periods of life Folia morph 23 102–111, 1964

40 *Sommers, S C* Endocrine changes with prostatic carcinoma. Cancer *10* 345–358, 1957

41 *Stieve, H* Mannliche Genitalorgane In Mollendorff, W von Handbuch der mikroskopischen Anatomie des Menschen Vol 7, part 2 Springer, Berlin 1930 p 1–399

42 *Teem, M van Buren* The relation of the interstitial cells of the testis to prostatic hypertrophy J Urol *34* 692–713, 1935

43 *Tillinger, K G, Birke, G, Franksson, C* & *Plantin, L O* The steroid production of the testicles and its relation to number and morphology of Leydig cells Acta endocr *19* 340–348, 1955

44 *Tveter, K J* & *Attramadal, A* Selective uptake of radioactivity in rat ventral prostate following administration of testosterone 1,2 ³H Acta endocr *59* 218–226, 1968

45 *Vermeulen, A, Rubens, R* & *Verdonck, L* Testosterone secretion and metabolism in male

senescence J Clin Endocr *34* 730 735, 1972

46 *Wendel, E F, Brannen, G E, Putong, P B & Grayhack, J T* The effect of orchiectomy and estrogens on benign prostatic hyperplasia. J Urol *108* 116-119, 1972

47 *Young, H H, II & Kent, J R* Plasma testosterone levels in patients with prostatic carcinoma before and after treatment J Urol *99* 788-792, 1968

Acta path microbiol scand Section A 81 3, 315–322, 1973

HEART PATHOLOGY IN CHRONIC ALCOHOLISM

JENS HOGNESTAD and PER TEISBERG

From the Institute of Forensic Medicine, University of Oslo Norway

In an autopsy material consisting of 35 alcoholics and 13 non alcoholic traffic casualties the heart was made the object of a thorough prospective study The investigation of the histological sections was performed blindly In two thirds of the alcoholic hearts changes were found which could not be explained by mechanisms other than chronic ethanol intoxication The coronary arteries showed the same degree of atherosclerosis in both groups Three alcoholics died suddenly without any alcohol in blood or urine probably from acute alcoholic cardiomyopathy It is stressed that the combination of heart hypertrophy, subendocardial interstitial, and perivascular fibrosis and the presence of chronic inflammatory cells is highly indicative of alcoholic heart disease The entity is probably more common than generally accepted

The association between alcoholism and heart disease is controversial Both alcohol addiction and heart disease are *per se* common in the western hemisphere, and the simultaneous occurrence of the two states in one patient does not establish a causal relationship Although almost 100 years have passed since *Bollinger* in his description of the 'Munchener Bierherz' (4) drew attention to the possible toxic effect of ethanol on the heart, both clinicians and pathologists still have difficulties in classifying the alcoholic heart as a clinical entity

[illegible] a co-hol and who quite suddenly develops shortness of breath, palpitation, fatigue and weakness X ray examination reveals generalized cardiomegaly and ECG is abnormal Haemodynamic measurements show left failure with low cardiac output Thiamine therapy is of no value and if drinking is not stopped the prognosis is poor (2)

Even if alcohol is withdrawn the size of the heart rarely returns to the normal At autopsy the heart is usually grossly enlarged, occasionally with mural thrombi, and microscopy reveals a diffuse fibrosis and muscular hypertrophy

The discovery of the Beri beri heart (1) early focused attention on vitamin B deficiency as the underlying factor in alcoholic heart disease In Beri beri, however, the patient usually exhibits other signs of malnutrition such as oedema of the legs, ascites, and pericardial effusion The cardiovascular insufficiency is of a hyperkinetic type with increased cardiac output, and low peripheral vascular resistance The heart is enlarged Thiamine treatment should in a few days normalize the clinical picture, and the heart reverts to normal size, in a few weeks

The toxic effects of ethanol on the heart

Received 2 viii 72 Accepted 29 xii 72

Requests for reprints should be addressed to Prosektor *Jens Hognestad*, Rettsmedisinsk Institutt, Rikshospitalet Oslo 1, Norway

now seem to be well established, both from pathophysiological studies in man (14, 16), pathology reports (5, 8, 11, 16, 18), and animal experiments (6, 10, 15)

Alcoholics are prone to develop malnutrition and B vitamin deficiency, and it is evident that the clinical picture may be confusing if vitamin B deficiency and the toxic effects of alcohol act together

A possible relationship between alcoholism and atherosclerosis has been widely discussed (7) There is at the present time no reason to believe that alcoholics have less atherosclerosis than non alcoholics (12)

In forensic medical practice, the question whether alcoholic heart disease may cause sudden unexpected death often arises The object of the present study was to perform a *prospective and controlled investigation of* heart pathology in an autopsy material of Norwegian alcoholics

Two questions were asked which pathological lesions prevail in the hearts of chronic alcoholics, and to what extent can a reliable diagnosis of ' alcoholic heart disease be based on these criteria?

TABLE 1 *Age Distribution in the Two Groups*

Age group	Alcoholics	Non alcoholics
29–39	4	4
40–49	16	5
50–59	11	3
60–70	4	1
Total	35	13

MATERIALS AND METHODS

The material included the bodies of all males known to be alcohol addicts in the age group 29 70 years (Table 1) and investigated at the Institute of Forensic Medicine in the period October 1970 to September 1971 They were all found dead under such circumstances that the police required autopsies to be performed in order to rule out any criminal event Information regarding drinking habits were obtained from the police and from close relatives All of them had consumed excessive amounts of alcohol daily for at least 10 years The original material consisted of 39 individuals but 4 of these were omitted because of coronary occlusion with myocardial infarction Thirteen males in the same age group who died in trafficor industrial accidents comprise the control material There was no information and nothing indicated that any of these had been excessive drinkers Causes of death are listed in Tables 2 and 3

TABLE 2 *Cause of Death in 35 Alcoholics*

Hypothermia	2
Acute ethanol intoxication	13
Combined ethanol and drug intoxication	4
Cerebral contusion	6
Trauma of the chest	1
Acute pancreatitis	1
Pneumonia	2
Epilepsia	2
Pulmonary embolism	1
Acute alcoholic cardiomyopathy	3
	35

TABLE 3 *Cause of Death in 13 Controls*

Cerebral contusion	7
Carbonmonoxyde poisoning	1
Aortic rupture	1
Decapitation (by train)	1
Drowning	2
Multiple dilacerations	1
	13

Autopsy A full autopsy was in all cases performed between 5 and 24 hours after death Weight and length of the bodies were noted Histological sections from liver kidneys heart and brain were routinely studied Sections from other tissues were examined only if macroscopic examination revealed pathological changes Blood and urine were examined for alcohol content and in some cases further toxicological investigations were carried out

Examination of the heart The heart weight and gross pathological lesions in the pericardium, epicardium myocardium and the endocardium were noted The valves were examined for lesions

The degree of atherosclerosis in the coronary arteries was quantitated arbitrarily in three stages by naked eye evaluation

means no or negligible atherosclerosis
\+ means atherosclerosis moderate without narrowings
\+ + means atherosclerosis pronounced

Alcoholics
Non-alcoholics

Atherosclerosis

Hypertrophia

Cellular infiltration

Fibrosis stage 1

Fibrosis stage 2

0 10 20 30 40 50 60 70 80 90 100 %

Fig 1 Pathological changes in the hearts of 35 chronic alcoholics and in 13 non alcoholic individuals The frequency is given in per cent of the total number of individuals in each of the two groups

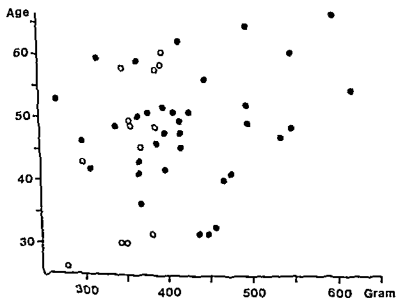


Fig 2 Heart weight and age of 35 chronic alcoholics (●) and 13 non alcoholic (○)

with narrowings
but no occlusions

Heart hypertrophy was defined as a heart weight exceeding 0 5 per cent of body weight by more than 100 gm (13)

Sections from each of the following areas were taken

- 2 sections from the left ventricle wall
- 1 section from the anterior part of the interventricular septum
- 1 section from the posterior part of the i v septum
- 1 section from the right ventricle

Histological preparation All specimens were fixed in Zenker Formol and embedded in paraffin Sections 4μ thick were cut and stained with Haematoxylin Eosin All sections were given random numbers

Light microscopy evalution The microscopical evaluation was done blindly i e the examiners made their description without knowing from which case the sections were taken

The degree of fibrosis was arbitrarily divided in two stages

Stage I slight interstitial fibrosis

Stage II marked perivascular and interstitial fibrosis together with small areas of localized fibrosis

RESULTS

The average degree of atherosclerosis was similar in both groups as judged by the naked eye appearance (Fig 1) The actual heart weights related to age in the two groups are listed in Fig 2

About two thirds of the hearts in the alcoholic group showed infiltration of inflamma

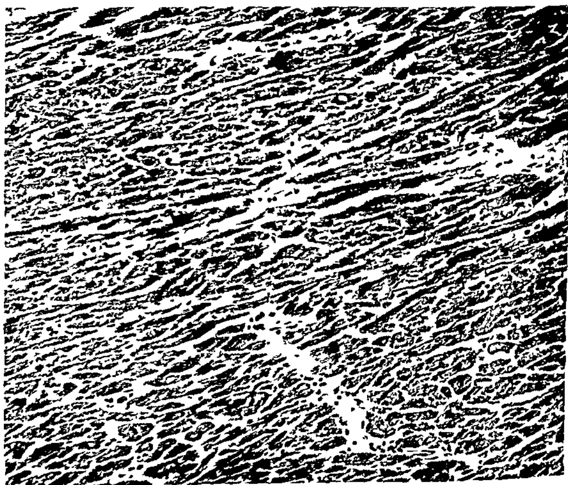


Fig 3 A slight diffuse infiltration of mononuclear cells in the left myocardial wall Age 57 Heart weight 500 gm Body weight 62 Kg Cause of death Acute ethanol intoxication × 360 H E stain



Fig 4 Heavy infiltration of mononuclear cells in the pericardial tissue Age 47 Heart weight 420 gm Body weight 82 kg Cause of death Acute ethanol intoxication × 144 H E stain

tory cells, mostly lymphocytes and plasma cells, but a few granulocytes also occurred occasionally The cellular infiltration was diffuse and of very moderate degree (Fig 3) In three cases, the pericardium and the underlying myocardium showed more massive signs of chronic inflammation (Fig 4) In *one case, a mural thrombus was found in the* right ventricle

Fibrosis was present in both groups, but the advanced degree of fibrosis classified as Stage II was only present in the alcoholic group (Fig 5) Fibrosis might occur in the subendocardial, interstitial and perivascular areas The predominant lesion was a diffuse interstitial fibrosis replacing only a few muscle fibres in each area (Fig 6)

In no instances were vacuoles indicating fat infiltration observed in the muscle fibres

Three cases, in which death was sudden and unexpected, need a comment One of the men had no alcohol in blood and 0 43 $_0/^{00}$ alcohol in the urine In the other two, no alcohol was found Their hearts were grossly hypertrophied with Stage II diffuse fibrosis and moderate cellular infiltration Scattered areas of necrosis of the myocardium were seen in all three individuals, and we presume that these three cases represent the acute fulminant alcoholic cardiomyopathy

Other pathological findings Thirty two alcoholics had various degrees of fatty infiltration of the liver Three had incipient cirrhosis, but no cases of overt cirrhosis were found

The examination of the kidneys revealed one case in the alcoholic group who had a *severe arteriolosclerosis* with a heart weight of 500 gm A moderate degree of arteriolosclerosis was found in 9 cases, but the heart weight did not exceed 0 5 per cent of the body weight in any of these

None of the brains showed signs of Wernicke's encephalopathy

DISCUSSION

The present investigation is based on an autopsy material and the post mortal autolysis has to be taken into account in the evaluation of the pathological changes We did not attempt to evaluate the possible acute toxic cellular effects of ethanol The use of myocardial biopsy in living patients is probably the only way by which information on these specific lesions is obtainable (2) Our parameters were easy to recognize the difficulty was to quantitate *these parameters*

Although all the individual lesions are unspecific, the combination of diffuse and patchy, perivascular, subintimal, and interstitial fibrosis, infiltration of inflammatory cells, and a large heart in the absence of significant atheromatosis seems to make an entity strongly indicating chronic alcoholism

It is an old conception that alcohol in moderate doses has a beneficial influence on the heart or the coronary flow Experimental data (10) has revealed increased coronary flow,



Fig 5 Interventricular septum with larger areas of fibrosis Age 50 Heart weight 325 gm Body weight 65 Kg Cause of death Subdural hematoma. × 360 H-E stain

but this was explained as a secondary phenomenon to increased oxygen consumption in the myocardium Others found no changes (17), or a decrease (20) in coronary flow Coronary atheromatosis in our alcoholic series was of the same extent and magnitude as in the control group

Fibrosis is an unspecific reaction to a variety of different agents, and is the end result of both inflammation and tissue anoxia Heart hypertrophy is, in the absence of congenital disease and acquired valve defects, an expression of heavy work load

The hypertrophy induced by alcohol may be the effect of a chronic process of degeneration and repair going on in the heart The muscle necrosis leads to scarring and fibrosis To *restore the contractile power* in the myocardium, each myocyte increases in size and the result is a generalized hypertrophy of the myocardium together with a generalized fibrosis

It might be *inferred that* vitamin deficiencies and malnutrition may have been the underlying factors causing heart disease in these patients, and that many of our alcoholic hearts may have been Beri-beri hearts However, Beri beri is a very rare condition in hospitalized alcoholics in Norway at present (19)

The toxic effects of alcohol upon the heart have been studied experimentally in animals In experiments with chronic alcohol-intoxicated rats, *Maines* (15) found a markedly depressed isometric systolic tension, and an ade-

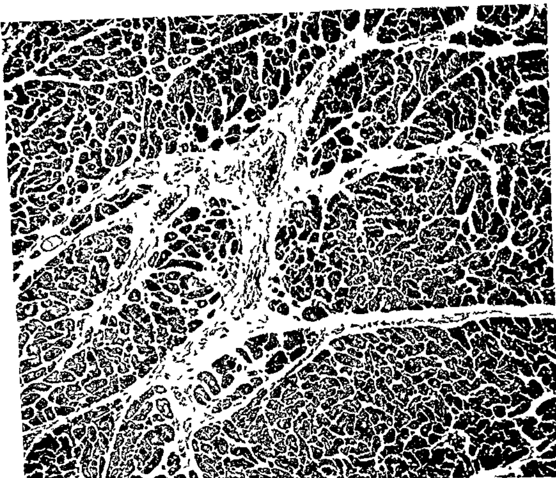


Fig 6 Posterior interventricular septum with perivascular and interstitial fibrosis Age 32 Heart weight 480 gm Body weight 87 kg Cause of death Acute ethanol intoxication × 144 H E stain

quate supplement of vitamin B did not protect against a decrease in myocardial contractility *Wendt* (21) showed release of oxidative enzymes from the myocardium of alcoholic patients, and *Ferrans (9)* concludes that these observations indicate a process of widespread myocardial degeneration with an end result of cell death and healing by fibrosis

Various authors (16) have proposed an increased vascular permeability in alcoholics They have suggested that proteinrich plasma accumulates between the layers of the arteriolar walls producing gradual narrowings and distortion of the vessel lumen If the endocardium also has an increased permeability this may lead to a subendocardial fibrosis and, secondarily, to the formation of mural thrombi The vascular changes may progress to degeneration of the wall with occlusion of the lumen This is followed by small foci of myocardial necrosis and later on by scarring

The occurrence of widely distributed chronic inflammatory cells also points to a chronic degenerative process in the heart These cells are among the important characteristics of the morphological diagnosis.

Varying degrees of fatty infiltration were seen in all alcoholic livers while no sign of liver abnormality was observed in the control group

The absence of overt liver cirrhosis in our

material may possible be explained by the fact that the cases are selected, and that the patients who develop cirrhosis will gradually fall ill and die in hospital *Sundby* (19) has moreover found that cirrhosis of the liver is less frequent in Norway than in other western countries

The result of the present investigation support earlier studies which have indicated that alcohol induces a pathological process in the myocardium The presence of heart hypertrophy, interstitial fibrosis and cellular infiltration seem to be the morphological correlate of the alcoholic heart

REFERENCES

1 *Aalsmeer, W C* & *Wenckebach, K F* Herz und Kreislauf bei der Beri Beri Krankheit Wien Arch inn Med *16* 257–271, 1929

2 *Alexander, C S* Electron microscopic observations in alcoholic heart disease Brit Heart J *29* 200–206, 1967

3 *Alexander, C S* Alcohol and the heart Ann Int Med *67* 670–674, 1967

4 *Bollinger, O* Ueber die Haufigkeit und Ursachen der idio-pathischen Herzhypertrophie in Munchen Deutsche Med Wchnschr *10* 180 181, 1884

5 *Brigden, W* & *Robinson, J* Alcoholic heart disease Brit med J *2* 1283–1289, 1964

6 *Burch, G E*, *Colcolough, H L* *Harb, J M* & *Tsui, C Y* The effect of ingestion of ethyl alcohol wine and beer on the myocardium of mice Amer J Cardiol *27* 522–529 1971

7 *Cabot R C* The relation of alcohol to arteriosclerosis J Amer Med Ass *43* 774–775, 1904

8 *Evans W* Alcoholic cardiomyopathy Amer Heart J *61* 556–567, 1961

9 *Ferrans, V J*, *Hibbs, R G*, *Weilbaecher, D G*, *Black, W C*, *Walsh, J J* & *Burch, G E* Alcoholic cardiomyopathy Amer Heart J *69* 748–765, 1965

10 *Ganz, V* The acute effect of alcohol on the circulation and on the oxygen metabolism of the heart Amer Heart J *66* 494–497 1963

11 *Hibbs, R G*, *Ferrans, V J*, *Black, W C* *Weilbaecher, D G*, *Walsh, J J* & *Burch, G E* Alcoholic cardiomyopathy An electron microscopic study Amer Heart J *69* 766–779, 1965

12 *Hirst, A A*, *Hadley, G G* & *Gore, I* The effect of chronic alcoholism and cirrhosis of the liver on atherosclerosis Amer J Med Sci *249* 143–149, 1965

13 *Hudson, R E B* Cardiovascular pathology vol 1 E Arnold Ltd, London 1965

14 *Kerr, A* Myocardiopathy, alcohol and pericardial effusion Arch Intern Med *119* 617–619, 1967

15 *Maines, J E* & *Aldinger, E E* Myocardial depression accompanying chronic consumption of alcohol Amer Heart J *73* 55–63, 1967

16 *Pintar, K*, *Wolanskyj, B M* & *Gubbay, E R* Alcoholic cardiomyopathy Canad Med Ass J *93* 103–107, 1965

17 *Regan, T J*, *Moschos, C B* & *Hellemo H K* Ethanol induced alteration of myocardial metabolism and function (P) Circulation *28* 788 789, 1963

18 *Schenk, E A* & *Cohen, J* The heart in chronic alcoholism Clinical and pathologic findings Path Microbiol (Basel) *35* 96–104, 1970

19 *Sundby, P* Alcoholism and mortality Thesis Universitetsforlaget, Oslo 1967

20 *Webb, W R* & *Degerli, I U* Ethyl alcohol and the cardiovascular system J Amer med Ass *191* 1055–1058, 1965

21 *Wendt V E*, *Ajluni, R*, *Bruce T A*, *Prasad, A S* & *Bing, R J* Acute effects of alcohol on the human myocardium Amer J Cardiol *17* 804–812 1966

cta path microbiol scand Section A 81, 3, 323–336, 1973

ON THE SIDE-EFFECTS OF CONTRAST MEDIA FOR MYELOGRAPHY

A Histological Examination of the Spinal Cord, Nerve Roots and Meninges after Experimental Myelography with Iodophendylate and Methiodal

JOHANNES KLITGAARD JAKOBSEN

Institute of Hygiene (Head Gunner J Bonde), Aarhus University, Denmark,
Department of Neuropathology (Head Edith Reske Nielsen)
and Department of Neuroradiology (Head Erling Ratjen), Aarhus Municipal Hospital
Aarhus University, Denmark

Iodophendylate (Pantopaque®) was injected into the spinal subarachnoid space of 56 rats, and a histological examination of the spinal cord, nerve roots and meninges was performed after 1, 4 8 16, 30 60 and 120 days Bacteriological and roentgenological controls were made Histological alterations, which in the subgroups seemed very similar, were found in all rats A moderate unspecific inflammation of the meninges, resulting in a state of fibrous scarring after 120 days was seen The meningeal reaction was a little enhanced after injection of blood and iodophendylate but after 120 days any quantitative differentiation between the two groups could not be made The endoneurium of the nerve roots displayed a fibrosis parallel to the changes in the meninges Within the first month an increasing loss of neurones in the spinal cord was observed By reducing the injected amount of contrast media the injuring effect on the grey matter was avoided Ninety rats were myelographied with methiodal (Conturex®) in a suboccipital position Fifty five died after a few minutes. The remaining rats all showed a slight generalized perivascular extravasation of erythrocytes and an acute vacuolization and extreme swelling of the neurones in the grey matter After 8 days, the number of nerve cells had decreased and any further alterations of the neurones were not noticed The changes in the meninges were slight and did not differ from those of the rats injected with physiological saline

As a means of visualizing the subarachnoid space for purposes of human myelography, iodized oils have been used since 1921, and water soluble iodine compounds have been employed since 1931

Several different contrast media have been in use in the years since then, which is a measure of the not negligible risk of complication involved in myelography with positive contrast media. Our knowledge of the harmful effects of the contrast media depends mainly on clinical observations and experimental experiences are few The side-effects have by most authors been ascribed to the meningeal reaction and experimental studies have as a rule been preoccupied with conditions in the meninges

There is, however, clinical and experimental support for assuming a harmful effect

Received 13 x.72 Accepted 22 xii 72

Requests for reprints should be addressed to *Johannes Klitgaard Jakobsen* Department of Neuropathology Aarhus Municipal Hospital DK 8000 Aarhus C Denmark

on the spinal cord or the nerve roots After methiodal myelography, cramps have been observed, electrophysiological investigations have shown decreased conduction velocity, and histological changes in the white matter have been described Persistent radicular pains, paresis and disturbances of the bladder function following myelography with iodophendylate have been reported

This paper was therefore planned as an experimental histological investigation of both the spinal cord and the nerve roots, as well as of the meninges in animals after myelography with the iodized oil iodophendylate (Pantopaque®) and the water soluble methiodal (Conturex®)

MATERIALS AND METHODS

The material consists of 100 male Wistar rats which at the start of the experiment had a body weight of 250 ± 40 g and an age of 13 weeks ± 1 week

Iodophendylate (Pantopaque® s g 1,26) is a mixture of isomeric esters of which the principal constituent is ethyliodophenylundecylate which in the spinal fluid is hydrolysed into ethyl alcohol and undecylenic acid, the latter forming a sparingly soluble soap with the calcium ion (21) Methiodal (Abrodil®, Conturex®, Kontrast U®, Methiodal® and Sciodan® s g 1,030) is a 20 per cent solution of a sodium salt of mono-iodomethyl sulphurous acid (1)

The contrast medium was injected suboccipitally during Nembutal anaesthesia, the rat being placed on a specially constructed table, its head inflected and fixed A pilot study involving injection of a dye, using a needle with a cutting angle of 45 per cent, assured that the medulla oblongata was not traumatically damaged The rat was then suspended by a suture in the region of the neck for a period of one hour whereby the contrast was concentrated in the lumbosacral region of the subarachnoid space This was checked roentgenologically

Iodophendylate myelography was performed on 57 rats an extradural deposition of the contrast medium was seen only in one case on the X ray pictures. In the days immediately after myelography the presence of contrast was shown all along the spinal cord being located around the nerve roots but concentrated cervically and lumbosacrally One and four months later, iodophendylate was situated lumbosacrally and in the posterior fossa of the skull It was not possible roentgenologically to demonstrate the presence of contrast medium in the subarachnoid space after use of the less radiopaque, water soluble contrast medium methiodal, but the tonic spasms and the inflected hindlegs were such constant and characteristical phenomena that the location of the contrast could be estimated clinically

In earlier experimental studies of animals inflammatory changes in the meninges have been found to develop after myelography with either methiodal or iodophendylate For this reason, culture of bacteria from injection fluids needles and syringes was made at the start of the experiment and also antemortally by withdrawing a few drops of spinal fluid by the described suboccipital technique The propagation media used were a solution of 1 per cent peptones, dextrose bouillon and a thioglycollate medium In three cases the presence of bacteria was shown in the antemortal culture but there was no quantitative or qualitative difference in the histological changes if compared with findings in other animals in the same group, no bacteria could be demonstrated in the glycerine sections and so the pollution has been regarded as accidental

TABLE 1 *The Table Shows the Number (No) of Rats Killed at Different Intervals (Int) after the Injection of Iodophendylate into the Subarachnoid Space*

	Group I A	Group I B	Group I C
Int (days)	No of rats	No of rats	No of rats
1	3	–	–
4	3	3	–
8	3	–	–
16	3	3	–
30	3	3	–
60	3	–	–
120	16	10	3

Group I A rats injected with 0 15 ml iodophendylate
Group I B rats injected with 0 075 ml iodophendylate with the admixture of 0 075 ml blood
Group I C rats injected with 0 015 ml iodophendylate

After study of the literature and some pilot experiments the following subgroups were established (Table 1 and 2) the stress being placed on the acute changes in the methiodal animals and the later changes in the iodophendylate animals

Since a special purpose of this paper has been to study the action of contrast media on the spinal cord, a dose was chosen which in relation to the weight of the spinal cord of each individual rat was

of the same size as the doses used in the clinical routine The size of the dose was fixed at 0 15 ml (Group 1A) The chosen technique did not permit

scribed a stronger effect on meninges after admixture of blood Group 1C was included because about 9/10 of the contrast medium is withdrawn after the myelography procedure in man

TABLE 2 *The Table Shows the Number (No) of Rats Killed at Different Intervals (Int) after the the Injection of 0,15 ml Methiodal into the Subarachnoid Space*

	Group II A	Group II B
Int	No of rats	No of rats
10 min	–	2
6 hours	3	–
1 day	5	–
4 days	5	–
8 days	6	–
16 days	3	–
30 days	3	–
90 days	8	–

Group II A rats suspended by a neck suture after the methiodal injection
Group II B rats suspended by the hindlegs after *the methiodal injection*

All the rats in group 2 were injected with 0,15 ml methiodal In this experiment, methiodal gave rise to extravasation of erythrocytes of capillaries in the spinal cord Two rats (Group 2B) *were for this* reason suspended by the hind legs after the injection and killed 10 minutes later, since extravasation *in the sacral section of the spinal cord* pointed to a general vascular effect, while absence of extravasation would make a local effect more probable

Group 3A serves as control by which to determine whether histological changes occurred in untreated rats in the course of the period of experiment The group consists of two rats, 3 months old, and two other rats, 7 months old Group 3B is the actual control group of the experiment, since these rats have been through exactly the same experimental procedure as the animals injected with iodophendylate and methiodal

The five rats of group 3B were injected with 0,15 ml physiological saline in the subarachnoid space and killed 1 4, 8 16 and 30 days after the injection

The animals *from all the groups were* killed by exvisceration during ether anaesthesia. Immediately after each rat had been tied to a corkboard, the upper half of the skull was removed and total laminectomy, down to the coccygeal vertebrae, was performed according to the method developed by *Reske-Nielsen* (1961) The rat was fixed in 4 per cent formalin and, after a period of 24 hours, the *brain and the spinal cord were extricated* The spinal cord was divided into a cervical, a thoracic, a lumbar and a sacral part and cauda equina which were kept separate during the further procedure Each of these parts were cut into four pieces of equal size, so that twenty different sections were to be evaluated in the histological examination The brain was cut into four equally thick parts

The specimens were dehydrated in alcohol, embedded in paraffin, cut into sections 7 microns thick and stained with haematoxylin and eosin, van *Gieson, toluidine blue and Weil-Weigert (staining*

cyanin chromalum, Einarson), lipid (Scharlach rot) and elastin (Weigert)

In the twenty sections of the spinal cord, the white and the grey matter, the meninges, the nerve roots and the ganglia have been evaluated on the basis of the following qualitative criteria Acute oedema, congestion, fresh or older extravasation *of erythrocytes, changes in the walls of the vessels,* infiltration by polymorphonuclear cells, infiltration by mononuclear cells, infiltration by phagocytes, connective tissue proliferation, status spongiosus in the white matter, reaction of glial cells, degeneration of myelin sheaths and degeneration of nerve cells.

For each rat, 247 qualitative evaluations have thus been performed and quantification of these have been attempted in the following way

Grade 0 The histological appearance of untreated rats (Group 3A)
– 1 Very slight changes
2 Slight changes
– 3 Moderate changes
– 4 Massive changes

The results for each rat are tabulated In each subgroup, similar histological changes were found in all the rats without exception The quantitative evaluation within the same time period never showed a variation of more than one grade in the description of qualitatively identical changes localized in the same region A check on the quantitative graduation was made by a randomized evaluation of ten rats which gave *variations of maximally* one grade for each single estimation The histological description has been made on the basis of the tables *The results pertaining to the brains* have not been tabulated

Fig 1 Pantopaque spaces in the thickened arachnoid membrane 16 days after the injection of iodophendylate and blood (H & E × 300)

Fig 2 Pantopaque spaces 16 days after the injection of iodophendylate In one end of the space to the right, some lipid positive material has remained (arrow) Opposite in the same Pantopaque space a phagocyte is seen close to a fat droplet (Scharlach rot × 500)



Fig 3 Anterior horns from the sacral part of the spinal cord, 30 days after the injection of iodophendylate In the right horn only a few nerve cells are preserved compared with the left horn Note the gliosis. (Toluidine blue × 125)

RESULTS

I Iodophendylate (Pantopaque®)

Immediately after the injection of the contrast medium there were no clinically observable changes in the Nembutal anaesthetized rats During the next 2 3 days, however, the animals showed signs of stiffness of the neck and they were noticeably inactive, concurrently, the intake of food was reduced by about 50 per cent.

The macroscopic appearance of the meninges and the spinal cord did not differ from that observed in non myelographied rats (Group 3A)

Group 1A In the *leptomeninges*, a transient, slight oedema (grade 2) with slight diffuse infiltration of polymorphonuclear cells (grade 2) was seen after one day In the groups 4 days to 30 days, a growing slight to moderate (grade 2-3) infiltration with lymphocytes phagocytes, a few eosinophilic leucocytes and macrophages with lipid positive granules could be observed After 120 days there was still a slight cellular infiltration, but the number of eosinophilic leucocytes had increased From the 8th day, *proliferation of* fibroblasts and the formation of collagenous fibrils (grade 2) were noted, the latter lying in parallel and rather irregular coats and in several places situated around so-called "Pantopaque spaces' (Fig 1) The ' Pantopaque-spaces are circular or oval colourless spaces of various sizes which are surrounded by phagocytes with lipid positive granules in the cytoplasm In certain places, an amorphous homogenous material giving positive lipid reaction can be observed at the edge of the colourless space (Fig 2) After 30 days the connective tissue proliferation predominated (grade 3) over the cellular infiltration and after 120 days focal cicatricial changes in the arachnoid and fibrous thickening of the ligamentum denticulatum could be noticed

In all subgroups the meningeal reaction

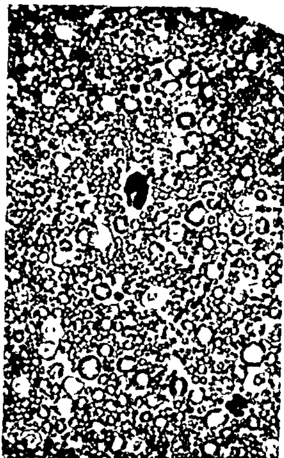


Fig 4 Nerve root from the cauda equina 30 days after the injection of iodophendylate Degenerated myelin sheaths and axis cylinders are seen (Weil Weigert × 500)

increased until one grade in the caudal direction and was further accentuated around nerve roots and vessels In 3 out of 16 animals intimal proliferations in the anterior spinal artery were observed after 120 days

In the *white matter* in the spinal cord a slight to moderate diffuse status spongiosus (grade 2 3) was seen throughout the experimental period The glial cell reaction was at a peak on the 8th day when the cells were swollen and proliferating with long forked offshoots Later the glial cell reaction was of a regressive nature

The number of affected neurones in the *grey matter* was steadily increasing during the first month (from grade 2 the 8th day to grade 2 4 after 30 days) Vacuolized cells with one or more small and peripheral vacuoles could be seen as well as shrunken cells and neurones surrounded by glial cells From the 8th day, loss of the nerve cells could be observed, also satellitosis was noted particulary situated in the anteromedian cellgroup of the anterior horns and most marked at lumbosacral sites (Fig 3) One anterior horn was often more heavily attacked than the other in the same specimen After 120 days there were many shrunken nerve cells (grade 2 3 cervicothoracally and grade 3-4 lumbosacrally) surrounded by glial cells and nerve cell debris together with a diffuse gliosis

Everywhere in the spinal cord the endothelial cells of the capillaries were in a state of proliferation from the fourth day of the experiment and throughout the rest of the experimental period The myelin sheaths of the *nerve roots* showed slight to moderate pathological changes from the 8th day They were seen to be swollen having differing dimensions and a noticeably reduced stainability, particularly pronounced in the cross sections of the cauda equina (Fig 4) The axis cylinders were sometimes seen to be swollen and collier like, exentrically situated in the myelin sheaths Furthermore proliferation of the Schwann cells was noted after eight days Running parallel to the changes in the meninges were very slight infiltrates in the perineurium (grade 1 2) In both endo- and perineurium a slight to massive progressive fibrosis (grade 2 4) was seen after 16 days (Fig 5 and 6) All changes in the nerve roots were most significant caudally

In the *ganglia* slight infiltration and fibrosis could be observed peripherally and from the 8th day the nerve cells showed very slight to slight (grade 1 2) changes with increased satellitosis

The brains After 30 days macroscopic examination showed a slight diffuse symmetrical dilation of the ventricular system The microscopic examination of the brains showed changes in the meninges, as described in the preceding and most pronounced corresponding to the base of the skull Slight degenerative changes were found in the nerve cells in the hippocampus and in the basal ganglia

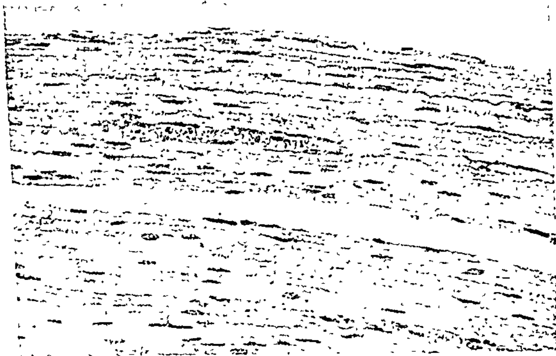


Fig 5. Nerve root from the cauda equina, 30 days after the injection of physiological saline. Only a few collagene fibrils are seen. (Van Gieson, × 300, green filter).



Fig 6 Nerve root from the cauda equina, 120 days after the injection of iodophendylate. Note the massive endoneurial fibrosis (Van Gieson, × 300, green filter)



Fig 7 Infiltration of the leptomeninges of the cauda equina 4 days after the injection of iodophendylate and blood (H & E × 300)

Group 1B After 4 days the infiltration in the leptomeninges (Fig 7) was moderate to massive (grade 3 4) in the sacral part and *in cauda equina being slight to moderate* (grade 2 3) around the other parts of the spinal cord The fibroblasts were proliferating (grade 1 2) after only 4 days when newly formed 'Pantopaque spaces could be seen Focal cicatricial changes in the arachnoid membrane (grade 3 4) were seen after 120 days but there was no longer any demonstrable difference in the strength of the reaction as compared to group 1A

In the white matter, an initial gliosis in the periphery of the sacral part could be seen after four days, but apart from this neither quantitative nor qualitative differences in the spinal cord and the nerve roots in animals in groups 1A and 1B have been observed (Fig *10*)

Group 1C The changes in the leptomeninges after 120 days were qualitatively and quantitatively of the same character as those in groups 1A and 1B In the grey matter *there were only very slight to slight changes* (grade 1 2) of the nerve cells (Fig 10)

2 Methiodal (Conturex®)

A fast, fine tremor of ears and whiskers was observed a few seconds after injection and, 10 15 seconds later, a violent spastic contraction of the facial and thoracic musculature Artificial respiration was required and after 1 minute, the thoracic spasm was replaced by a clonic state 60 per cent of the rats injected with Conturex developed the



Fig 8 Anterior thoracic horn 4 days after the injection of methiodal Note the swollen nerve cells (appears as holes) (Toluidine blue × 125)

Cheyne Stoke respiration and died with indications of *pulmonary oedema*, a foaming sanguinolent fluid issuing from nose and mouth The surviving rats were suspended by the suture of the neck Gradually a kyphosis of co - lumna set in and a simultaneous flexion of the hindlegs was observed The femora were drawn up towards the abdomen

In several rats belonging to the early sub groups, slight haemorrhages were seen macroscopically and, in one rat severe bleeding at the clivus Microscopic examination showed several small perivascular extravasations in all examined structures from all examined rats, but in a few of the animals larger haemorrhages were seen in the brain stem, the cerebellum or the cervical part of the spinal cord, often invading the spinal canal

Group 24 In the leptomeninges, only a slight oedema (grade 1 2) was seen in the group after 6 hours, but small, dispersed haemorrhages were found in all groups (grade 1 3) There was a very slight to slight (grade 1-2) diffuse cellular infiltration, at first involving polymorphonuclear cells, later lym *phocytes and phagocytes On the 8th day and* throughout the rest of the experiment, a very slight to slight (grade 1-2) fibrosis in the leptomeninges was seen All the changes were most pronounced cervically In the early sub groups, focal metachromasia was seen in the wall of the anterior spinal artery After 30 *days, the internal elastical layer had the appea* rance of a fragmented, swollen and reduplicated membrane In nine out of the eleven longest surviving rats in this group intimal proliferations were noticed

In the *white matter,* status spongiosus could, throughout the period of the experi*ment, clearly be seen to be located in the* periphery and slightly to moderate (grade 2 3) cervicothoracically, but only rarely sacrally After 90 days, foci of pronounced status spongiosus were found, separated by areas of white matter of normal appearance The progressive glial cell reaction culminated on the fourth day The cells were *swollen, prolifera* ting, vacuolized, carrying gritty, clearly demarcated offshoots, which a times encased the



Fig 9 Three swollen nerve cells from the cervical part of the spinal cord, 4 days after the injection of methiodal The ballooning of the neurones extends into the axons (arrows) (Toluidine blue, × 500)

myelin sheaths From the 8th day, the regressive glial cell changes augmented and, after *30 days, nearly all cells were pyknotic* From the fourth day on, proliferation of the endothelial cells of the capillaries were observed in both white and grey matter

In the *grey matter,* slightly dilated capil laries and small perivascular extravasations of erythrocytes were noted after six hours After *one day some of the nerve cells showed slight* degenerative changes (grade 1 2) with low stainability and excentrically situated nuclei. The cytoplasm was occupied by very fine and closely packed vacuoles which at times melted together form larger ones In the group of 4 days, massive changes in the nerve cells have been observed in the cervical part (grade 4) and, with decreasing intensity, caudally (grade 1 3) Most nerve cells were enormously swollen and balloon like with extreme vacuolization (Fig 8) The vacuoles had va rying sizes, and they stretched out to form dendrites and axons, where they have a dia *meter of 10 20 microns At times the vacuoles* melted together to form giant vacuoles the diameters of which might be up to 100 mic rons, they were situated near the nucleus (Fig 9) The nuclei were shrunken, deplaced and in some cells they had vanished from sight The glial cells were proliferating and frequently *found to be arranged in rosettes* around the nerve cells which only appeared as shadows After 8 days there was a moderate drop in the

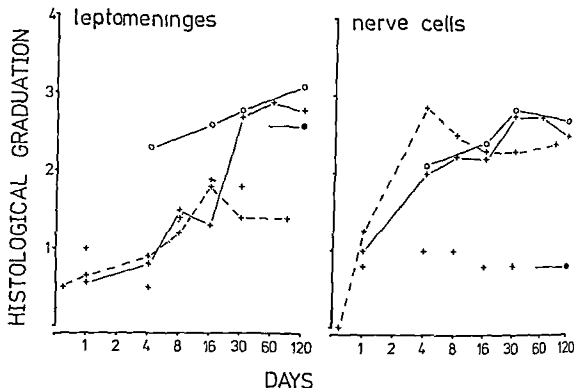


Fig 10 Histological alterations as regards leptomeninges and nerve cells after injection of different media into the subarachnoid space (+ —— + 0 15 ml iodophendylate ● —— ● 0 015 ml iodophendylate ○ —— ○ 0 075 ml iodophendylate and 0 075 ml blood + + 0 15 ml meth odal and + + 0 15 ml 0 88 per cent NaCl) The values of the histological graduation are means of *the quantitative estimations along the whole of the* spinal canal

number of neurones (grade 3) which did not accentuate in the following groups The loss of nerve cells was particulary located to the anteromedian cellgroups in the cervicothoracic part and was only slightly pronounced in the lumbar part At 90 days a diffuse gliosis most pronounced in the anterior horns, was observed

In the *nerve roots* slight changes in the myelin sheaths and a perivascular extravasation of erythrocytes were noted but there was no fibrosis or cellular infiltration The *ganglia* had their usual apperance

The brains Microscopic examination of the brains disclosed changes in the meninges and the vessels as described above In the brain stem vacuolization of the nerve cells to the same extent as in the cervical part of the spinal cord was noted In the cortex and the hippocampus, shrunken cells with long easily visualized axons and dendrites were seen after the first day of the experiment (grade 2) but conditions had returned to normal after 3 months

Group 2B In these two rats perivascular extravasations in the sacral parts of the spinal cord and the meninges were noted

3 *Control Group*

Rats which were injected with physiological saline were without symptoms after 12 hours None of the animals presented macroscopically observable changes

Group 3A The microscopic examination shows a certain range of distribution which in this study is regarded as the normal over all picture (grade 0)

Group 3B The leptomeninges showed very slight to slight cellular infiltration with mononuclear cells (grade 1 2) and a slight fibrosis

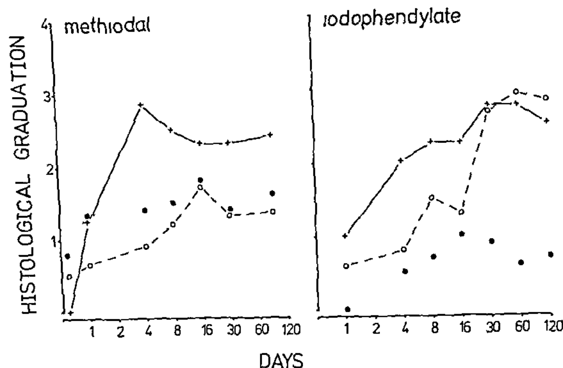


Fig 11 Histological alterations as regards inject on of 0 15 ml methiodal and 0 15 ml iodophendylate (+——+ nerve cells o o leptomeninges and ● ● vessels) The values of the histo log cal graduation are means of the quant tati e estimations along the whole of the spinal canal

was observed (grade 1 2) after 30 days In the white matter a very slight to slight status spongiosus was noted (grade 1 2) and in the grey matter, very slight degenerative changes (grade 1) with a few dark and vacuolized neurones were observed *initially (Fig 10)* There was a very slight to slight glial cell re action in both the white and the grey matter (grade 1 2) Roots and ganglia were of a nor *mal appearance*

DISCUSSION

It *is* a well known fact that PHYSIOLOGICAL SALINE and Ringer s solution provoke an irri tative meningeal reaction in the subarach noid space (7, 21) as demonstrated in ani mals in the control group in this *study* The very slight changes in the grey and white matter in animals in the control group is pos sibly also caused by physiological saline, since traumatic damage to the spinal cord could not be shown in the pilot study, an anoxic state of the rats *is not likely either since no* anoxic nerve cells have been found (16) However, it cannot with sufficient certainty be excluded that the reaction in the medul lary parenchyma *in animals in the control* group is a consequence of the conditions of the chosen technique It is for this reason that changes in groups 1 and 2 which did not *quantitatively surpass or qualitatively differ* from the findings in group 3B have not been ascribed to the contrast media

In rats injected with IODOPHENDYLATE the *irritative aseptic changes in the meninges and* the nerve roots are predominant, and the ad mixture of blood causes a quicker and stron ger reaction which, however after 4 months *cannot be quantitatively distinguished from* the histological appearance of rats which were injected with iodophendylate only The mye lin sheath degeneration and the changes in the grey matter are at a peak after a time of

one month when a moderate loss in the number of nerve cells is noted The latter is dependent on dosage and is not found in rats myelographied with relatively small amounts of iodophendylate

It is not probable that the pathogenesis of the changes in the grey matter depends on vascular mechanisms, since the changes in vessels were small and variable It is remarkable that the damage to the medullary parenchyma observed in this study coincides with the reactive changes in the meningeal structures of the spinal canal (Fig 11) and both reach a peak at the same time (1 month) This makes it probable that the myelin sheath and nerve cell changes are secondary to the fibrosis in the endoneurium, the perineurium and the meninges But the dependence on the size of the dose, which does not seem to include the meningeal structures, points to a toxic effect, possibly caused by the product of saponification (21)

In earlier experimental studies of the action of iodophendylate on meninges (12, 13, 14, 17, 18, 21, 22, 23, 25), changes have been found which seem to correspond to those described in this paper In the chronically fibrotic stage, the presence of infiltrates with eosinophilic leucocytes was assumed (12), but the authors had no opportunity to observe these since the experimental period did not go beyond seven weeks

Emulsification of the oil soluble contrast medium iodophendylate entails strong reactive changes in meninges and the emulsification is stabilized by bloodserum (15) The effect of iodophendylate with admixture of blood was studied by *Howland and Curry* (1966) who found a meningeal reaction stronger than the one seen in dogs myelographied by means of contrast medium without admixture of blood This finding is confirmed by the present study but the difference can no longer be demonstrated after a period of four months The nerve roots have not been histologically examined by other authors, and the changes noted will not be discussed further

Steinhausen et al (1944) saw cysts in the grey matter in the caudal part of the spinal cord in dogs after iodophendylate myelography, while *Fischer* (1965) in one of six cats found "aseptic' micro-abscesses In a very thorough histological study involving trepanation and injection of 0 1 ml Pantopaque in each of six cats, *Schober* (1964) found no noticeable (auffallenden) reaction in the grey and white matter of the brain This is in quite good accordance with the very slight changes found in the brains of the rats in the present study What is more, *Schober* employed a dosage which in relation to total weight was six times smaller than the one given to the rats

There exist quite a few reports on experiences obtained in experiments on animals with Lipiodol® (2, 3, 4, 5, 19) which was the preferred oil soluble contrast medium for myelography in the years 1925 45 Lipiodol (iodized poppyseed oil) is hydrolysed in the spinal fluid and the free fatty acid saponifies together with the calcium ion, in this respect it resemples iodophendylate In fact, the irritative effect on the meninges of, on the one hand, Lipiodol, on the other Pantopaque, can be neither qualitatively nor quantitatively distinguished from each other (12) Degenerative changes in the grey matter have been described by several authors (2, 3 5, 19) after Lipiodol myelography on dogs and rabbits Dissolution of Nissl substance as well as excentric localization of the nuclei, vacuolization of cytoplasm, shrunken and lost nerve cells have been observed The degenerative nerve cell changes are probably dependent on the amount of contrast medium used (4, 19) The amount, relative to total weight at which changes in the nerve cells could no longer be observed, is of the same approximate size in *Peiper* and *Klose's* paper (1925) as in the present study

In the METHIODAL group, the acute nerve cell changes predominate The universal small

[illegible]
could not be distinguished from the appearance in the meninges of the control group

animals (group 3B) were found, and this accords well with the findings by other authors (9, 12, 21)

The experiences from experiments with methiodal are few, the clinical reactions of animals vary, some authors have mixed the contrast medium with anaesthesia, and thus, attempts at a comparison of results obtained in this study with earlier experiences demand great caution

Schober (1964) observed subpial oedema and glial cell reaction in the brain, but no nerve cell changes The amounts of contrast media employed were, however, relatively small, as described above *Funkquist and Obel* (1961) found foci of oedema and necrosis with structural disorganization of the white matter of the spinal cord, as well as oedema of the myelin sheath, glial cell proliferation and swelling of the axons The changes were most marked peripherally in the spinal cord and were noted early in the experimental series The contrast medium contained anaesthesia, but conditions in the grey matter have not been communicated, which makes comparison difficult The changes in the white matter observed in this study however, seem to be in good agreement with the findings by *Funkquist and Obel* (1961)

Initial rises in blood pressure after injection of methiodal have been described (8, 11) *Funkquist and Obel* (1960) found a rise in blood pressure up to an average of 290 mm Hg against the normal 190 mm Hg This rise in blood pressure could be completely avoided by addition of spinal anaesthesia, as in the routine in methiodal myelography on human beings The vascular damage and the acute perivascular extravasations found in the present study could conceivably be caused by an initial rise in blood pressure triggered by the brain stem The fact that the rats of group 2B showed extravasations in the sacral part of the spinal cord without any direct contact with the contrast, speaks for this hypothesis

The damage to the nerve cells in they grey matter could stem from the initial vascular changes (Fig 11) If so, the degenerative neurone changes could very likely have been avoided if the spinal anaesthesia had been employed for the myelography

The severely swollen nerve cells are, however, morphologically identical with the changes provoked by acute intoxication (akute Schwellung), as described by *Nissl and Spielmeyer* (1922) and *Greenfield (1960)* According to the opinion of the present author, this implies a toxic pathogenetic mechanism of the nerve cell changes rather than a vascular pathogenetic mechanism This toxic quality of methiodal could be ascribed to the hyperosmolarity (9), ensuing from the use of the contrast medium

---

This work was performed as a prize paper under the University of Copenhagen, and was supported by grants from *Lœge Else Poulsens Mindelegat, Danish Medical Research Council, Fonden til Lœgevidenskabens Fremme* and a scholarship under the *University of Aarhus*

I want to thank mrs *E Horn* for excellent technical assistance

REFERENCES

1 *Arnell, S* Myelography with water soluble contrast Acta Radiol, suppl. 75, Stockholm 1948

2 *Boldrey, E* & *Aird, R B* The effect of iodized poppyseed oil and iodine chlorine in peanut oil in the subarachnoid space of animals J Nerv Ment. Dis. *99* 521–533, 1944

3 *Bruskin J* & *Propper, N* Experimentelle Myelo-encephalographie an Hunden und uber den Einfluss von Jodipin und Lipojodol auf das Ruckenmark Gehirn und dessen Haute Z ges exp med *75* 34–55 1931

4 *Craig R L* Effect of iodized poppyseed oil Neurol

5 " The spinal subarachnoid space J A M A *94* 772–777, 1930

6 *Fisher, R L* An experimental evaluation of pantopaque and other recently developed myelographic contrast media Radiology 85 537–545 1965

7 *French, J D* Clinical manifestations of lumbar spinal arachnoiditis. Surgery *20* 718–729, 1946

8 *Funkquist, B* & *Obel, V* Tonic muscle spasms and blood pressure changes following the subarachnoid injection of contrast media Acta radiol *53* 337–352, 1960

one month when a moderate loss in the number of nerve cells is noted The latter is dependent on dosage and is not found in rats myelographied with relatively small amounts of iodophendylate

It is not probable that the pathogenesis of the changes in the grey matter depends on vascular mechanisms, since the changes in vessels were small and variable It is remarkable that the damage to the medullary parenchyma observed in this study coincides with the reactive changes in the meningeal structures of the spinal canal (Fig 11) and both reach a peak at the same time (1 month) This makes it probable that the myelin sheath and nerve cell changes are secondary to the fibrosis in the endoneurium, the perineurium and the meninges. But the dependence on the size of the dose, which does not seem to include the meningeal structures, points to a toxic effect, possibly caused by the product of saponification (21)

In earlier experimental studies of the action of iodophendylate on meninges (12, 13, 14, 17, 18, 21, 22, 23, 25), changes have been found which seem to correspond to those described in this paper In the chronically fibrotic stage, the presence of infiltrates with eosinophilic leucocytes was assumed (12), but the authors had no opportunity to observe these since the experimental period did not go beyond seven weeks

Emulsification of the oil soluble contrast medium iodophendylate entails strong reactive changes in meninges and the emulsification is stabilized by bloodserum (15) The effect of iodophendylate with admixture of blood was studied by *Howland and Curry* (1966) who found a meningeal reaction stronger than the one seen in dogs myelographied by means of contrast medium without admixture of blood This finding is confirmed by the present study, but the difference can no longer be demonstrated after a period of four months The nerve roots have not been histologically examined by other authors, and the changes noted will not be discussed further

Steinhausen et al (1944) saw cysts in the grey matter in the caudal part of the spinal cord in dogs after iodophendylate myelography, while *Fischer* (1965) in one of six cats found "aseptic" micro-abscesses In a very thorough histological study involving trepanation and injection of 0 1 ml Pantopaque in each of six cats, *Schober* (1964) found no noticeable (auffallenden) reaction in the grey and white matter of the brain This is in quite good accordance with the very slight changes found in the brains of the rats in the present study What is more, *Schober* employed a dosage which in relation to total weight was six times smaller than the one given to the rats

There exist quite a few reports on experiences obtained in experiments on animals with Lipiodol® (2, 3, 4, 5, 19) which was the preferred oil soluble contrast medium for myelography in the years 1925 45 Lipiodol (iodized poppyseed oil) is hydrolysed in the spinal fluid and the free fatty acid saponifies together with the calcium ion, in this respect it resemples iodophendylate In fact, the irritative effect on the meninges of, on the one hand Lipiodol on the other, Pantopaque, can be neither qualitatively nor quantitatively distinguished from each other (12) Degenerative changes in the grey matter have been described by several authors (2, 3 5, 19) after Lipiodol myelography on dogs and rabbits. Dissolution of Nissl substance as well as excentric localization of the nuclei, vacuolization of cytoplasm, shrunken and lost nerve cells have been observed The degenerative nerve cell changes are probably dependent on the amount of contrast medium used (4, 19) The amount relative to total weight, at which changes in the nerve cells could no longer be observed, is of the same approximate size in *Peiper* and *Klose's* paper (1925) as in the present study

In the METHIODAL group the acute nerve cell changes predominate The universal small perivascular extravasations of erythrocytes

[illegible]
could not be distinguished from the appearance in the meninges of the control group

Acta path microbiol scand Section A *81, 3*, 337–343, 1973

ANTIGEN-STIMULATED DNA SYNTHESIS IN THE BURSA OF FABRICIUS OF THE CHICKEN

Rune Back

Department of Histology, University of Uppsala, Uppsala, Sweden

The influence of antigenic stimulation on cell proliferation in different lymphoid organs of the chicken was investigated, as studied by ^3H thymidine incorporation into DNA Six-week old chickens were either immunized with human serum albumin (HSA) or given sterile physiological saline 24 or 72 hours before sacrifice Thirty minutes before sacrifice, the chickens were intravenously labelled with ^3H thymidine. At 24 hours after the immunization the incorporation of label (cpm/μg DNA) into the bursa of Fabricius was significantly higher in the immunized chickens than in the saline injected controls. At 72 hours after the immunization, the mean incorporation of label into the bursa was of the same magnitude in the immunized and the control chickens These findings suggest that the bursa of Fabricius, although a central lymphoid organ, may react by an increased cell proliferation early after antigenic stimulation The incorporation of label into the thymus was not at any time influenced by the immunization

It is now well established that the thymus in mammals and the thymus and the bursa of Fabricius in birds are essential for the normal development and expressions of immunological functions (6, 29, 36) It has been demonstrated that these central lymphoid organs seed other lymphoid organs with cells during the ontogeny of the lymphoid system (10, 20, 21, 31, 37) Also a thymus humoral factor (38) may be of importance for the normal development of immunologically competent cells in other lymphoid organs

There is still little information concerning early cellular events in the thymus and the bursa of Fabricius following antigenic stimulation The central lymphoid organs are not considered directly involved in immune reactions because neither the thymus nor the bursa seem to produce specific antibodies after systemic immunization (8) Furthermore, the thymus and the bursa are developed normally in germ free animals, in constrast to the quantitatively reduced peripheral lymphoid tissues (34) These findings and the normal thymus weight of mice subjected to prolonged antigenic stimulation (28) suggest that cell proliferation in the central lymphoid organs is autonomous and not affected by antigenic stimulation (29)

There is however, some evidence that cells in the thymus may respond to antigenic stimulation *Fichtelius et al* have demonstrated an increased DNA-synthesis in the thymus of normal (12) and adrenalectomized (13) rats following pertussis vaccination, the latter has also been shown by *Lundin* (25) in hypophysectomized rats immunized with pig serum More recently an increased RNA-synthesis (5) and an increased ribonuclease activity (27) in the thymus after immunization with sheep erythrocytes have been reported

Investigations have also shown that the

Received 22 xi 72 Accepted 3 xi.72

Requests for reprints should be addressed to Rune Back, Department of Histology, Vastra Agatan 26 A S-752 20 Uppsala, Sweden

normal traffic patterns of transfused (11) and *in situ* (22, 24) labelled mammalian thymus cells are changed after immunization (24) and after sensitization with dinitrochlorobenzene (DNCB) (11, 22) In the chicken, an increased homing of thymus derived cells to the bone marrow (2) and of bursa-derived cells to the spleen (3) have been shown 3 days after immunization with human serum albumin (HSA) It is thus possible that cells in the thymus and in the bursa of Fabricius respond to antigenic stimulation and that this is reflected in their pattern of migration to other lymphoid organs However, no clear-cut data on the influence of antigen on bursa cell proliferation using DNA precursors seem to exist Therefore such a study seemed highly warranted

The present investigation was designed to study the influence of immunization with human serum albumin (HSA) on cell proliferation, as studied by ^{3}H-thymidine incorporation into DNA in the bursa of Fabricius and other lymphoid organs of chickens in the early stages of the immune response

MATERIAL AND METHODS

Animals and Experimental Design

Newly hatched, White Leghorn cockerels of the Babcock *B 300* strain were obtained from a local vendor and raised with free access to standard chicken feed and water At the age of six weeks they were matched by weight into four groups

One group of fifteen chickens body weight 435–570 grams was immunized with one intravenous injection of 40 mg/kg body weight of human serum albumin (HSA 100 per cent pure Mann Research Laboratories New York USA) 23 5 hours before the intravenous administration of ^{3}H thymidine into a wing vein (lot number 620–041, spec act 6 7 Ci/mM, New England Nuclear Corp Boston, Mass, USA) A group of fourteen control chickens body weight 435 570 grams received 1 ml of sterile p [illegible] 23 5 ho r before the intraven[illegible]

Anoth[illegible]

440 565 grams, was [illegible] cribed above, 71 5 hours before the intravenous administration of ^{3}H thymidine (lot number 620–119) A group of thirteen controls body weight 445–555 grams received 1 ml of sterile physiological saline 71 5 hours before the intravenous administration of ^{3}H thymidine

All animals received 0 5 mCi/kg body weight of the ^{3}H thymidine as calculated from the indicated vial content All labelling was performed between 9 a m and 1 p m

Thirty minutes after labelling with ^{3}H thymidine the chickens were killed by an intravenous injection of *5 ml of air* The thymus, bursa of Fabricius, spleen, caecal tonsils, bone marrow from the left femur and duodenum were sampled for the radiochemical studies The seven thymus lobes on the left side, the bursa of Fabricius the spleen and the caecal tonsils were carefully dissected and immediately weighed on a Mettler type H4 balance (Mettler Instrumente AB, Greifensee Zurich, Switzerland), and the organ weight index $\frac{\text{organ weight}}{\text{body weight}} \times 10^3$) was calculated for each organ Then samples from the same organs were taken for subsequent radiochemical analyses

Radiochemical Studies

The organ samples were immediately immersed into 5 ml of cold 5 per cent trichloroacetic acid (TCA) and frozen The extration of nucleic acids was performed using a modified (20) *Schneider* (33) technique The organ samples were homogenized in 5 ml of cold 5 per cent TCA with a Potter Elvehjem homogenizer The homogenates were centrifugated at 4° C at 2000 × g for 10 minutes The supernatants were discarded and the precipitates washed twice with 5 ml of cold 5 per cent TCA Using this procedure the residue contains the nucleic acids and the discarded supernatant the acid soluble compounds among which tritiated water is the most important The nucleic acids were extracted in 5 per cent TCA in a water bath at 90° C for 30 minutes The DNA concentrations of the supernatants were measured with Burton s modification of the diphenylamine reaction at 600 nm using a Beckman B spectrophotometer DNA from sperm (Nutritional Biochemicals Corp Cleveland Ohio USA) was used as standard reference solutions Ribonucleic acids and lipids were not separated from DNA since these compounds do not disturb the diphenylamine reaction (4)

The radioactivity was measured in a Packard Tri Carb liquid scintillation counting system One ml samples of nucleic acids in 5 per cent TCA were mixed with 10 ml of Insta Gel® scintillator

(26) Each sample was counted twice and the

mean of these values, after background subtraction, was used to calculate the specific activity counts per minute per μg DNA (cpm/μg DNA), of the different organ samples. The means of the specific activities of the different organs of the HSA immunized and the saline injected non-immunized chickens were compared with Student's t test.

The distribution of label between the different organs was also calculated. The *percentual activity* of each organ sampled was determined, i.e.

$$\frac{\text{Specific activity of an organ} \times 100}{\text{Sum of the specific activities of all sampled organs in the same animal}}$$

RESULTS

Radiochemical studies

The specific activity values (cpm/μg DNA) of the different organs at 24 hours and 72 hours after the administration of HSA or saline are given in Table 1 and Table 2. At 24 hours (Table 1), the specific activity of the bursa of Fabricius of the immunized chickens (70±2) was significantly higher ($p<0.02$) than the specific activity of the bursa of the non-immunized controls (62±2). The values for the thymus, spleen, caecal tonsils, bone marrow and duodenum were not significantly changed by the immunization at this time.

At 72 hours (Table 2), the specific activity of the bursa was of the same magnitude in

TABLE 1. *Specific Activity* (Means±S.E.M.) of Different Organs of Six Week Old Chickens Given HSA or Saline 24 Hours and ³H Thymidine§ 30 Minutes before Sacrifice*

	HSA n = 15†	Saline n = 14	P
Thymus	18±1	17±1	—
Bursa of Fabricius	70±2	62±2	p<0.02
Spleen	123±6	113±5	—
Caecal tonsils	68±3	63±3	—
Bone marrow	134±7	132±9	—
Duodenum	117±7	113±8	—

* Specific activity = cpm/μg DNA
§ Lot No. 620-041
† Number of animals

TABLE 2. *Specific Activity* (Means±S.E.M.) of Different Organs of Six Week Old Chickens Given HSA or Saline 72 Hours and ³H Thymidine§ 30 Minutes before Sacrifice*

	HSA n = 13†	Saline n = 13	P
Thymus	22±1	21±1	—
Bursa of Fabricius	78±5	78±4	—
Spleen	135±6	127±5	—
Caecal Tonsils	77±3	66±3	p<0.02
Bone Marrow	150±10	141±7	—
Duodenum	122±8	119±8	—

* Specific activity = cpm/μg DNA
§ Lot No. 620-119
† Number of animals

the immunized (78±5) and in the control chickens (78±4). On the other hand, the incorporation of the caecal tonsils in the immunized chickens (77±3) was significantly higher ($p<0.02$) than in the controls (66±3). The spleen values were higher for the immunized chickens than for the controls both at 24 and 72 hours, but neither difference reached a statistically significant level. The specific activities of the bone marrow and duodenum were not apparently influenced by the antigenic stimulation.

The comparison between the values in Tables 1 and 2 reveals that the means of the specific activity values for all organs were lower in the 24 hour groups of animals than in the 72 hour groups. However, due to a possibly lower spec. act. of the ³H thymidine in the lot used for the 24 hour groups than in the one used for the 72 hour groups (J. Mc Mahan, New England Nuclear Corp., personal communication), the organ specific activity values (cpm/μg DNA) for the 24-hour and 72 hour animals are not directly comparable. Therefore, the distribution of label between the different organs, the *percentual activities*, was calculated. These values (Table 3) would not be influenced by minor differences in the isotope dose administered. The distribution of label between the different organs, as revealed by these

TABLE 3 *Percentual Activities* (Means±S E M) of Different Organs of Six Week Old Chickens Given HSA or Saline 24 or 72 Hours and ³H Thymidine 30 Minutes before Sacrifice*

	24 hrs		72 hrs	
	HSA	Saline	HSA	Saline
Thymus	3 4±0 2	3 3±0 2	3 6±0 3	3 8±0 3
Bursa of Fabricius	13 3±0 6	12 4±0 6	13 4±0 8	14 0±0 6
Spleen	23 1±0 8	23 5±1 0	23 7±1 4	23 1±0 8
Caecal Tonsils	13 0±0 5	12 6±0 3	13 1±0 7	11 9±0 3
Duodenum	21 9±0 9	22 3±0 9	20 2±1 4	21 5±1 1
Bone Marrow	25 1±0 7	26 4±1 3	25 7±1 6	25 5±1 0

* *Percentual activity* = $\frac{\text{Spec. act. of an organ} \times 100}{\text{Sum of spec act of all sampled organs in the same animal}}$

values, did not differ significantly between the different groups of animals The mean of the percentual activity of the bursa of the immunized chickens at 24 hours was higher than that of the saline injected 24 hour chickens, and the same applies to the value for the caecal tonsils of the immunized chickens at 72 hours as compared to the non immunized controls However, these differences did not reach the levels of statistical significance

Organ Weights

At 24 hours, no effect of the immunization on the organ weight indices could be observed (Table 4) At 72 hours, the mean spleen weight index was significantly higher ($p < 0.005$) in the immunized chickens (2 41±0 15) than in the controls (1 83±0 11) Also the mean bursa weight appeared to be higher in the immunized chickens (5 00±0 30) than in the saline injected controls (4 19±0 27), but the difference was not statistically significant The organ weight of the thymus and the caecal tonsils were not changed at 24 or 72 hours after the antigenic stimulation

DISCUSSION

The results of the present investigation demonstrated a significant difference in the incorporation of ³H thymidine into the bursa of Fabricius in HSA immunized chickens and non immunized controls 24 hours after administration of HSA and saline as revealed

TABLE 4 *Organs Weight Index* (Means ± S E M) for Different Lymphoid Organs of Six Week Old Chickens Given HSA or Saline 24 or 72 Hours before Sacrifice*

	24 hrs		72 hrs		
	HSA	Saline	HSA	Saline	P
Thymus§	2 98±0 10	2 96±0 12	2 87±0 19	2 87±0 19	—
Bursa of Fabricius	4 06±0 21	4 37±0 25	5 00±0 30	4 19±0 27	p<0 10
Spleen	2 19±0 06	2 05±0 12	2 41±0 15	1 83±0 11	p<0 005
Caecal Tonsils	0 28±0 03	0 25±0 02	0 21±0 02	0 23±0 02	—

* Organ weight index = $\frac{\text{organ weight}}{\text{body weight}} \times 10^3$

§ The seven thymus lobes on the left side

by the specific activity values (cpm/μg DNA) for this organ This difference could also be seen in the percentual activity values although it did not reach statistical significance

The specific activity values for all organs in the 24 hour groups were lower than those in the 72 hour groups Furthermore if the means of the percentual activity values for the different organs in the saline treated animal groups are compared the values for the bursa and the thymus appeared to the lower in the 24 hour group than in the 72 hour group but the differences were not statistically significant The possibility remains open in spite of the strict standardization of the experimental conditions, that the difference in the incorporation of ^{3}H thymidine in the immunized and non immunized 24 hour group could have been due to stress (14) or other factors beyond experimental control Hence the conclusion could not be reliably drawn that the significantly higher specific activity of the bursa of the HSA immunized chickens at 24 hours as compared with the saline injected controls would be caused by an increased proliferation of bursa cells after antigenic stimulation

Another factor complicating the interpretation of the data is the radioactive precursor itself While other techniques have shown that cell proliferation in the thymus is very rapid (18) the incorporation of ^{3}H thymidine into the thymus is low (7) The present data confirmed this observation This discrepancy has been attributed to a large pool of desoxyribosyl compounds in the thymus (32) Little is known about this pool in the bursa but its function as a central lymphoid organ with rapid cell proliferation favours the assumption that such a pool may exist in the bursa In this study the incorporation of ^{3}H thymidine into the bursa was about 4 fold the incorporation into the thymus but lower than that in other sampled organs Thus the pool of desoxyribosyl compounds may be smaller in the bursa than in the thymus although not negligible It could therefore not be completely ruled out that the increased incorporation of label into the bursa of the immunized chickens has been caused by changes in the pool of intermediates between the injected precursor and DNA, or by other factors affecting the availability of the ^{3}H thymidine in the bursa The incorporation of label was not influenced in the thymus however where the pool of desoxyribosyl compounds may be larger

With these reservations, the fact remains that the specific activity (cpm/μg DNA) of the bursa samples was significantly higher in the immunized chickens than in the non immunized controls at 24 hours after the immunization One explanation of this finding is that the bursa reacts to antigenic stimulation by an increased cell proliferation This view has some support in the fact that the mean bursa weight in the immunized chickens at 72 hours is higher than that in the saline injected 72 hour group, although the difference was not statistically significant Furthermore *Abramoff and O Brien* (1) have reported a rapid increase of pyronnophilic cells of the plasmacytic cell line 13 hours after immunization with sheep erythrocytes Maximal numbers of such cells in the bursa were found at 48 hours but in the spleen not until 72 hours after the immunization The appearance of these pyronnophilic cells may be associated with an increased bursa cell proliferation

It is also possible that the antigenic stimulation changed the composition of the bursa cell population Bursa cells are derived from blood borne progenitor cells (30) and there is also a normally occurring cell traffic from the bursa to other lymphoid organs (15 16) Thus if the immunization initiated an export of mainly non dividing cells from the bursa and/or an immigration of proliferating lymphoid precursor cells the proportion of proliferating cells could have increased 24 hours after the antigenic stimulation

Apart from the work of *Abramoff and O Brien* (1) there is little information on the capacity of the bursa to respond to antigenic stimulation It has been demonstrated that bursa cells synthesize immunoglobulins

(17, 35), and that a small proportion of the lymphocytes in the embryonic bursa are antigen binding cells (9) However, chicken bursa cells did not produce specific antibodies after repeated immunization (8)

In a recent investigation (3), an increased number of ³H-thymidine labelled bursa derived cells was found in the spleen 3 days after HSA immunization This was explained by a recruitment of bursa derived cells to the spleen and/or an increased cell export from the bursa of Fabricius If the present results reflect a true proliferative response of the bursa, it is possible that the bursa reacts to antigenic stimulation by an increased cell proliferation followed by an increased cell export to the spleen

At 72 hours after the antigenic stimulation, the specific activity of the bursa was not changed above control levels However, the incorporation of label into the caecal tonsils was increased and there was also an increase in the mean organ weight of the spleen of the immunized chickens These observations are consistent with previous findings of a proliferative response of peripheral lymphoid tissues following antigenic stimulation (19)

The present investigation suggests, but does not prove, that bursa cells respond by an increased cell proliferation early after antigenic stimulation The data also suggest that the proliferative response is over 72 hours after the antigenic stimulation Studies are in progress to elucidate more definitely the possibility of an early proliferative response in the bursa of Fabricius after antigenic stimulation, using other nucleic acid precursors and other sampling times

This study was designed to elucidate the proliferative capacity of lymphoid organs after stimulation of humoral, bursa-dependent immunity It is therefore not surprising that no antigen induced change was observed in the thymus Similar studies, designed to challenge mainly thymus dependent immunity would be necessary to clarify whether thymus cells have the capacity to react by proliferation upon adequate antigenic stimulation

This work was supported by grants from the *Swedish Medical Research Council* (projects no B71 12X 33 07C and B71 12X 3155 01), the *Medical Faculty of the University of Uppsala* and the *P A and A A Petterson Fund*

I wish to thank Dr *T J Linna* and Dr *G J Alm* for valuable discussions during the preparation of the manuscript, and Miss *Lili Anne Ericsson* for competent technical assistance

REFERENCES

1 *Abramoff, P* & *O Brien, N B* Studies of the chicken immune response I Correlation of the cellular and humoral immune response *J Immunol 100* 1204–1209, 1968

2 *Back, R* & *Linna, T J* Influence of antigenic stimulation on lymphoid cell traffic in the chicken I Increased homing of thymus-derived cells to the bone marrow after antigenic stimulation Int Arch Allergy In press

3 *Back, R* & *Linna, T J* Influence of antigenic stimulation on lymphoid cell traffic in the chicken III Increased homing of bursa derived cells to the spleen following immunization Eur J Immunol In press

4 *Burton, K* A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA Biochem J *62* 315–323, 1956

5 *Cohen, E P Majer, J* & *Friedman, K* Responses to immunization in the thymus of the adult mouse J Exp Med *130* 1161 1174 1969

6 *Cooper, M D*, *Gabrielsen A E* & *Good R A* Role of the thymus and other central lymphoid tissues in immunolog cal diseases Annu Rev Med *18* 113 138 *1967*

7 *Craddock C G Nakai, G S Fukuta, H* & *Vanslager, L M* Proliferative activity of the lymphatic tissue of rats as studied with tritium labelled thymidine J Exp Med *120* 389–412, 1964

8 *Dent P B* & *Good, R A* Absence of antibody production in the bursa of Fabricius Nature (London) *207* 491–493 1965

9 *Dwyer J M* & *Warner, N L* Antigen binding cells in embryonic chicken bursa and thymus Nature New Biology (London) *229* 210–212 1971

10 *Ernstrom, U*, *Gyllensten L* & *Larsson, B* Venous output of lymphocytes from the thymus Nature (London) *207* 540–541, *1965*

11 *Fichtelius, K E* The influence on lymphocytes of sensitization to dinitrochlorobenzene shown by transfusion of labelled lymphocytes. Acta dermato venerologica *39* 75 81, 1959

12 *Fichtelius, K E, Gitlen, H & Hassler, O* On the mechanism of the lymphocytosis following pertussis vaccination Acta haemat *17* 106–110, 1957

13 *Fichtelius, K E & Hassler, O* Influence of pertussis vaccine on the lymphocyte production in adrenalectomized rats. Acta path microbiol scand *42* 189–192, 1958

14 *Glick, B* The experimental production of the stress picture with cortisone and the effect of penicillin in young chickens Ohio J Science *59* 81–86, 1959

15 *Hemmingsson, E J & Linna, T J* Ontogenetic studies on lymphoid cell traffic in the chicken I Cell migration from the bursa of Fabricius Int Arch Allergy *42* 693–710, 1972

16 *Hemmingsson, E J* Ontogenetic studies on lymphoid cell traffic in the chicken II Cell traffic from the bursa of Fabricius to the thymus and the spleen in the embryo Int Arch Allergy, *42* 764–774, 1972

17 *Kincade, P W & Cooper, M D* Development and distribution of immunoglobulin containing cells in the chicken J Immunol *106* 371 382, 1971

18 *Kindred, J E* A quantitative study of the haemopoietic organs of young albino rats Amer J Anat *67* 99–149, 1940

19 *Langevoort, H L* The histophysiology of the antibody response I Histogenesis of the plasma cell reaction in the rabbit spleen Lab Invest *12* 106–118, 1961

20 *Linna, T J* Transport of tritium labelled DNA from the thymus to other lymphoid organs in rabbits under normal conditions and after administration of endotoxin Int Arch Allergy *31* 313–337, 1967

21 *Linna, T J* Cell migration from the thymus to other lymphoid organs in hamsters of different ages Blood *31* 727 746 1968

22 *Linna, T J* Influence of contact allergy on thymus lymphoid cell migration Int Arch Allergy *38* 230–245, 1970

23 *Linna, T J, Back, R & Hemmingsson, E* Lymphoid cell migration *In* Morphological and Functional Aspects of Immunity K. Lindahl Kiessling, G Alm and M G Hanna Jr, eds. Adv exp med Biol vol 12 Plenum Press, New York 1971, p 149–159

24 *Linna, T J & Cohen, E P* Increased thymus cell traffic to the spleen in sheep erythrocyte- (SRBC) stimulated mice Fed proc 1972 In press.

25 *Lundin, P M* Cell proliferation in lymphoid tissue of normal and hypophysectomized rats after immunization with foreign protein Acta path microbiol scand *54* 59–65, 1962

26 *Lyttkens, L & Sjostrand, U* Dual isotope determination by liquid scintillation spectrometry *with special regard to samples* with varying quenching Beckman Instruments International S A, Geneva, 1971

27 *Maor, D & Witz, I P* Increased ribonuclease activity in the thymus and lymph nodes after intraperitoneal antigenic stimulation with sheep erythrocytes Immunology *20* 259–261, 1971

28 *Metcalf, D* Reticular tumors in mice subjected to prolonged antigenic stimulation Brit J Cancer *15* 769–779, 1961

29 *Miller, J F A P* Current concepts of the immunological function of the thymus. Phys Rev *47* 437–520, 1967

30 *Moore, M A S & Owen, J J T* Experimental studies on the development of the bursa of Fabricius Develop Biol *14* 40–51, 1966

31 *Nossal, G J V* Studies on the rate of seeding of lymphocytes from the intact guinea pig thymus Ann NY Acad Sci *120* 171 181, 1964

32 *Rotherham J & Schneider, W C* Deoxyribosyl compounds in animal tissues J Biol Chem *232* 853–858, 1958

33 *Schneider, W C* Phosphorous compounds in animal tissues I Extraction and estimation of *desoxypentose nucleic acid and of pentose* nucleic acid J Biol. Chem *161* 293–303, 1945

34 *Thorbecke, G J, Gordon, H A, Wostman, B, Wagner, M & Reyniers, J A* Lymphoid tissue and serum gamma globulin in young germ free chickens J Infect Diseases *101* 237–251, 1957

35 *Thorbecke, G J, Warner, N L, Hochwald, G M & Ohanian, S H* Immunoglobulin production by the bursa of Fabricius of young chickens Immunology *15* 123–134, 1968

36 *Warner, N L* The immunological role of the avian thymus and bursa of Fabricius Folia Biol (Praha) *13* 1–17, 1967

37 *Weissman, I L* Thymus cell migration J Exp Med *126* 291–304, 1967

38 *White, A & Goldstein, A L* Thymosin a thymic hormone influencing lymphoid cell immunologic competence *In* Hormones and the Immune Response G E W Wolstenholme and J Knight, eds. Churchill, London, 1970, p 3–23

Acta path microbiol scand Section A *81, 3,* 344-346, 1973

THE EFFECT OF LONG-TERM CHALLENGE WITH ENDOTOXIN ON THE GROWTH OF SAPPHIRE MINK WITH HIGH INCIDENCE OF INFECTIOUS PLASMACYTOSIS

KNUT NORDSTOGA and BJORN NESS

The National Veterinary Institute, and the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo, Norway

Young, growing female Sapphire mink presenting a high incidence of infectious plasmacytosis were injected with *Escherichia coli* endotoxin for a period of approximately 4 months The injections consisted of 1 mg each and the animals received a total of 29 inoculations. After 3 months of experimentation, there was no significant difference between the weights of the test animals and the control animals A slight positive effect of endotoxin treatment was observed towards the end of the experiment wl [illegible] nutritional conditions and received frozen feed and [illegible] cent of the experimental animals developed amyloido[illegible] endotoxin injected was not "toxic" in any respect other than its amyloidogenic action

In a previous experiment, in which the aim was to study whether repeated injections of endotoxin had an amyloidogenic effect in mink, the incidence of infectious mink plasmacytosis (IMP) was high (2) The endotoxin treatment did not seem to exert a negative influence on the health of these animals, as all 20 experimental young mink developed satisfactorily during the experimental period, whereas 2 out of 10 controls succumbed to IMP towards the end of the experiment The present investigation, comprising a considerably greater number of animals, was performed to investigate whether prolonged endotoxin challenge had any effect on the growth, development and health of young Sapphire mink in which a high incidence of IMP was suspected

Received 2 viii 72 Accepted 15 xi 72

Requests for reprints should be addressed to *Knut Nordstoga,* National Veterinary Institute, P B 8156, Oslo-Dep , Oslo 1, Norway

MATERIAL AND METHODS

The experiment involved 50 female Sapphire mink obtained from the same herd as the Sapphire mink in a previous investigation (2), in which IMP had been widely prevalent during recent years The animals were randomly allocated to the *experimental and control groups,* the only restriction being that two or more kits originating from the same litter were always distributed as equally as possible between the two groups The experiment started on August 11, 1970, when the animals were approximately 14 weeks old All animals were weighed on August 11, November 6 and December 15, the last time immediately after killing They were kept in individual pens, for the first part of the experiment in the disused barn at the Research Station for Fur Bearing Animals Heggedal, until November 23, when they were moved to the mink sheds where the other animals at the station were placed Both groups were maintained on the standard feed given to the other mink at the station

Escherichia coli 026 B6 endotoxin (Difco Laboratories Detroit, Mich USA) was dissolved in saline at a concentration of 2 mg/ml and frozen until used All animals in the test group received 29 subcutaneous injections of endotoxin, from

TABLE 1 *Body Weights of the Animals*

	Body weights (mean ± S D in grams		
	11/8 1970	6/11 1970	15/12 1970
Experimental group (24 animals)	730 6 ± 67 7	952 1 ± 77 3	915 2 ± 98 3
Control group (24 animals)	728 3 ± 72 2	911 7 ± 139 8	849 0 ± 108 8

August 11 to December 11 each challenge consisting of 1 mg given once or twice weekly the controls remained untreated Experimental and control animals were killed on December 15 Prior to killing blood samples were taken by heart puncture for the determination of plasma proteins The results of this investigation will be published elsewhere (1) Euthanasia was performed by cervical fracture Samples of various organs were fixed in 4 per cent neutral formaldehyde solution and sections from liver spleen and kidneys were stained with haematoxylin and eosin and with thioflavine T The thioflavine treated sections were examined as described previously (2) and the presence of a pale yellow fluorescence associated with homogenous deposits in liver and spleen was considered as specific for amyloid

RESULTS

Most animals seemed to develop normally during the experiment One test animal escaped during October, and one of the controls died during November, having been languishing for some days. Weights of animals were found to be significantly higher in the experimental group than in the control group ($p \leq 0\ 05$) at the end of the experiment (December 15), but not on November 6 and August 11 ($p > 0\ 05$) The mean weights of all animals in both groups and standard deviations are given in Table 1

Autopsy Findings

There were no obvious differences between the test animals and control animals at autopsy The spleen was slightly, but somewhat variably enlarged in several animals in both groups and a considerable splenomegaly was noted in two test mink and in two controls including the animal which died before the experiment was ended In these four mink, the liver and kidneys were also somewhat enlarged

Hepatic and renal lesions indicative of IMP were recognized in 7 experimental animals and in 8 control animals Two test animals as well as the control mink which died during the experimental period and an additional control showed advanced lesions of the liver and kidney suggestive of IMP Proliferations of plasma cells, of varying degree, were very common findings in both groups

Homogenous deposits identified as amyloid were found perifollicularly and/or around small arteries in the red pulp of the spleen in 14 test mink Seven of these animals exhibited also hepatic deposits of amyloid whereas storage of amyloid in the spleen was absent in one mink with hepatic amyloidosis Renal specimens did not reveal deposits of amyloid, nor was amyloid demonstrated in the organs of the controls, except for minor splenic storage in one mink with pronounced alterations indicative of IMP

DISCUSSION

The present investigation revealed that long term application of endotoxin to growing mink did not result in reduced body weight, as compared with untreated controls, although approximately 60 per cent of the test animals developed lienal and/or hepatic amyloidosis some of them with considerable storage of amyloid in the organs

The experiment extended and confirmed a previous report, showing that prolonged endotoxin challenge to mink leads to amyloidosis,

a response which is not significantly influenced by a simultaneous occurrence of IMP (2) One of the controls, severely affected by IMP, also exhibited minor splenic deposits of amyloid However, amyloid storage in various organs may also occur in long-standing cases of IMP (4, 5), and this finding may therefore be explained as a complication of IMP

When the animals were moved from the frostfree barn to the ordinary mink sheds towards the end of the experiment, the average weights of test animals as well as of control animals decreased This transfer in the late autumn, leading to poorer nutritional conditions, the animals receiving frozen feed and drinking water, may be interpreted as an extra strain on the animals It is a common experience that animals suffering from IMP frequently succumb under such environmental conditions As the weights of the experimental animals were significantly higher than the weights of controls after the transfer it may be assumed that the endotoxin treatment had enhanced their resistance in some way or other The amount of endotoxin (1 mg/animal once or twice weekly through 17 weeks) seemed to be appropriate, without toxic implications other than moderate amyloid formation

REFERENCES

1 *Mohn, S F* & *Nordstoga, K* In preparation
2 *Nordstoga, K* Amyloidosis in mink induced by repeated injections of endotoxin Acta path microbiol scand Section A *80* 159 168 1972
3 *Nordstoga, K* The effect of long term challenge with endotoxin on infectious mink plasmacytosis Acta path microbiol scand Section A *81* 347 351, 1973
4 *Obel, A L* Studies on a disease in mink with systemic proliferation of the plasma cells Amer J vet Res *20* 384 393 1959
5 *Trautwein, G* Experimentelle Untersuchungen uber die Aleutenkrankheit (Aleutian disease) der Nerze Arch exp Vet Med *18* 287–395 1964

Acta path microbiol scand Section A 81, 3, 347 351, 1973

THE EFFECT OF LONG-TERM CHALLENGE WITH ENDOTOXIN ON INFECTIOUS MINK PLASMACYTOSIS

KNUT NORDSTOGA

National Veterinary Institute, Oslo, Norway

Fifty female Sapphire kits were inoculated immediately after weaning with an organ suspension containing the agent of mink plasmacytosis The animals were equally divided into an *experimental group* and a *control group*, animals in the first group received injections of endotoxin originating from *Escherichia coli*, initially twice weekly and later once weekly, each dose consisting of 1 mg This treatment greatly enhanced the development of plasmacytosis and after 8 months of experimentation, only 3 animals in the test group survived, whereas 10 controls were still alive Most of the experimental mink revealed lienal and hepatic storage of amyloid, three animals had also amyloid deposits in renal glomeruli

Endotoxins can modify susceptibility to infections (2) A considerable number of the mink in two preceding experiments in which the animals received repeated injections of endotoxin, were affected by infectious mink plasmacytosis (IMP) and, as a slight beneficial effect of the endotoxin treatment on the resistance to IMP could not be excluded on the basis of the results obtained in these studies (17, 18), it was decided to investigate more thoroughly the possible effects of prolonged endotoxin challenge on IMP

MATERIALS AND METHODS

Fifty female Sapphire mink kits were purchased from a mink ranch immediately after weaning and equally divided into an *experimental* and a *control* group They were placed in one of the ordinary mink sheds at the Research Station for Fur Bearing Animals, Heggedal and fed the normal feed used for the non experimental animals at the Station Pairs of animals were kept together, i e one experimental and one control mink always shared a pen *Escherichia coli* 026 B6 endotoxin (Difco Laboratories, Detroit, Mich USA) was dissolved in saline at a concentration of 2 mg/ml and frozen until used The animals in the experimental group (i e all animals in Table 1 with odd numbers) received *subcutaneous injections of endotoxin* on June 25, 28 and 30, and on July 2 and 6

All animals in both groups were inoculated intraperitoneally on July 6, 1971 when they were approximately 8 weeks old, with 0 5 ml of a 10 per cent crude saline suspension of tissue (liver, spleen, kidney) from a spontaneous case of IMP

The endotoxin challenge was continued by one or two weekly injections of the same dose until *November 30*, later the animals received one weekly injection at regular intervals except that no treatment was given during the Christmas week Surviving animals were killed by cervical fracture on March 6 one week after the last challenge

Blood samples were collected on September 27, January 12 and March 6, for the estimation of plasma proteins The results of this investigation will be published elsewhere (14) The first 2 samples were taken from most animals by cutting the

Received 2 viii 72 Accepted 15 xi 72

Requests for reprints should be addressed to *Knut Nordstoga*, National Veterinary Institute, P B 8156, Oslo-Dep, Oslo 1, Norway

	Mink No	Died or killed, days after inoculation*	Month	Lesions indicating IMP**	Amyloid† liver	Amyloid† spleen	Number of injections (= total amount of endotoxin in mg)
Experimental group	1	170	Dec	+ + +	+ + +	+ + +	35
	3	37	Aug	+	–	+	11
	5	244	Mar	+ +	+ +	+ + +	44
	7	175	Dec	+ + +	+ + + +	+ +	35
	9	244	Mar	+ + +	+ +	–	44
	11	213	Feb	+ +	+ + +	+ + + +	40
	13	83	Sep	+ +	+	+ + +	19
	15	68	Sep	+	–	–	16
	17	80	Sep	+ +	+ +	+ + +	19
	19	232	Feb	+ + +	+ + +	+	43
	21	190	Jan	+ + +	+ + + +	+	37
	23	77	Sep	+ + +	+ +	+	18
	25	108	Oct	+ + +	–	+ + +	23
	27	202	Jan	+ + + +	+ +	+ +	39
	29	244	Mar	+ + + +	–	+	44
	31	85	Sep	+ + +	+	+ + +	20
	33	173	Dec	+ + +	+ + +	+ +	35
	35	137	Nov	+ +	+ + +	+ + + +	29
	37	170	Dec	+ +	+	+	35
	39	151	Dec	+ +	+	+	31
	41	81	Sep	+ + +	+ +	+ +	19
	43	135	Nov	+ +	+ + +	+ + + +	28
	45	139	Nov	+ +	+ +	+ +	29
	47	73	Sep	+ +	+ +	+ +	17
	49	104	Oct	+ + +	+ + +	+ +	22

* First day of experiment June 25, 1971 Surviving animals were killed on March 6, 1972

** Lesions were graded according to severity from + to + + + +, based on hepatic and renal changes

† – represents no storage, +, + +, + + + + + + + increasing deposits of amyloid

ventral artery of the tail while the last samples were taken by heart puncture, in association with the euthanasia

All animals were autopsied and pieces of tissue fixed in 4 per cent neutral formaldehyde solution Sections from liver, spleen, kidneys and adrenals were stained with haematoxylin and eosin selected sections also with methyl green pyronin Thioflavine-T stained sections were examined as described previously (17), the presence of yellow fluorescence associated with homogenous material in the liver, spleen and renal glomeruli was considered as specific for amyloid

RESULTS

No animals showed any symptoms after the inoculation with the organ suspension or endotoxin injections One control mink escaped and one animal in the same group died after accidental injuries during July (These two animals are not included in Fig 1) The first death, interpreted as death directly related to the experiment, occurred on August 12, 48 days after inoculation when mink No 3 died after it had received 11 injections of endotoxin Histopathological lesions indicating IMP, and moderate storage of amyloid in the spleen, were observed in this animal All animals to succumb later in the experiment, after they had received 16 to 43 injections of endotoxin, exhibited gross lesions compatible with IMP (i e enlargement of the spleen and lymph nodes, obvious renal changes), the diagnosis of IMP was in each

logical Lesions and Number of Injections

	Mink No	Died or killed, days after inoculation*	Month	Lesions indicating IMP**	Amyloid† liver	Amyloid† spleen	Number of injections (= total amount of endotoxin in mg)
Control group	2	192	Jan	+ +	–	–	
	4	244	Mar	–	–	–	
	6	190	Jan	+ + + +	–	–	
	8††						
	10	194	Jan	+ + +	+ + +	+ + +	
	12	244	Mar	+	–	–	
	14	86	Sep	+ +	–	–	
	16	244	Mar	+	–	–	
	18	202	Jan	+ + +	–	–	
	20	133	Nov	+ +	–	–	
	22§						
	24	132	Nov	+ + +	–	–	
	26	196	Jan	+ + +	–	–	
	28	244	Mar	+	–	–	
	30	244	Mar	+ +	–	–	
	32	120	Nov	+ + + +	–	–	
	34	188	Jan	+ + +	+ +	+ + +	
	36	131	Nov	+ + +	–	+ +	
	38	244	Mar	–	–	–	
	40	121	Nov	+ + +	–	–	
	42	244	Mar	+ +	–	–	
	44	244	Mar	+	–	–	
	46	244	Mar	+	–	–	
	48	244	Mar	–	–	–	
	50	138	Nov	+ + +	–	–	

†† This animal died after accidental injuries during July

§ This animal escaped during July

case confirmed by microscopical examination The 3 surviving animals in the test group were killed on March 6 and exhibited severe macroscopical and microscopical changes suggestive of IMP, whereas 3 out of 10 surviving controls were negative Most of the experimental mink had amyloid deposits in the spleen and/or liver (Table 1), 3 also in renal glomeruli (Nos 1, 11, 39) The difference in mortality in the test group and the control group is shown in Fig 1

DISCUSSION

The findings in this experiment offer substantial evidence that prolonged endotoxin treatment accelerates the development of IMP Endotoxins have diverse and ambiguous effects both on bacterial and viral infections, and on the immune response The effects of endotoxins are modified by a variety of factors, such as time of application in relation to exposure to infectious agents, dosage, mode of challenge, age and sex (2, 3–9, 13, 15, 16, 20, 21)

Endotoxin challenge has been shown to increase the primary antibody response in some species, in association with a protein antigen (10, 12) Although it has been convincingly demonstrated that IMP is a transmissible disease, the entity is regarded as a "slow virus infection", and auto immune mechanisms are

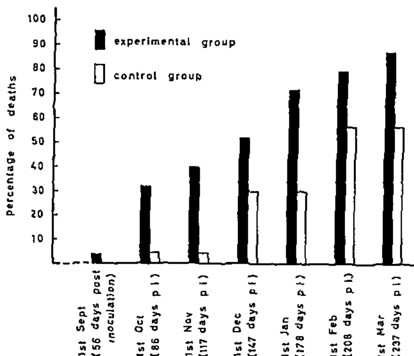


Fig 1 The mortality rates of the experimental and control groups. All animals were inoculated on July 6

considered to play an important role in the pathogenesis (1, 11, 19, 22)

Recently, it has been shown that immuno suppression is effective in preventing the progression of IMP (1) A survey of the literature has not revealed any previous reports on the effects of long term endotoxin treatment on a slow viral infection, or on an auto immune disease It has been claimed that repeated injections of endotoxin, prior to an antigenic stimulus, do not elevate the immune response in mice thus made tolerant to endotoxins (6)

The slight beneficial effect of endotoxin on the development of ' spontaneous" IMP, as assumed on the basis of previous experiments (17, 18) in which the animals probably had been exposed to variable, and moderate, amounts of the agent, may only have been transient, and could be interpreted as either an unspecific increase in resistance, or caused by a retardation in the development of the auto-immune disease In the present investigation, in which all animals received equal and probably more massive amounts of the agent, it can not be doubted that the prolonged endotoxin challenge had an accelerating effect on the development on IMP The dose of the infecting agent is known to be of some importance for the action of endotoxin upon the resistance to experimental infections (2) Hence, if compared with the present investigation the differences in the amount of the agent to which the animals were exposed in the preceding experiments (17, 18) may, at least in part, account for the different results Although the results to be obtained under other experimental conditions possibly might have been different, it seems unlikely that endotoxin treatment has any beneficial effect in connection with the development of this slowly progressive infectious disease A possible, correspondingly enhancing effect of ' exogenous" endotoxin on the progress of other slow viral infections and auto-immune diseases, remains to be evaluated

This study was supported by a grant from the Agricultural Research Council of Norway

REFERENCES

1 *Cheema, A, Henson, J B & Gorham, J R* Aleutian disease of mink Prevention of lesions by immunosuppression Amer J Path *66* 543–556, 1972

2 *Cluff, L. E* Effects of endotoxins on susceptibility to infections. J infect Dis. *122* 205–215, 1970

3 *Condie, R M, Zak, S J & Good, R A* Effect of meningococcal *endotoxin on resistance to* bacterial infection and the immune response of rabbits. Fed Proc. *14* 459–460, 1955

4 *Dubos, R J & Schaedler, R W* Reversible changes in the susceptibility of mice to bacterial infections I Changes brought about by injection of pertussis vaccine or of bacterial endotoxins J exp Med *104* 53–65, 1956

5 *Finkelstein, R A* Alteration of susceptibility of embryonated eggs to Newcastle disease virus by *Escherichia coli* and endotoxin Proc Soc exp Biol (N Y) *106* 481–484, 1961

6 *Freedman, H H, Nakano, M & Braun, W* Antibody formation in endotoxin tolerant mice Proc Soc exp Biol (N Y) *121* 1228–1230, 1966

7 *Gledhill, A W* Sparing effect of serum from mice treated with endotoxin upon certain murine virus diseases. Nature (Lond) *183* 185–186, 1959

8 *Gledhill, A W* The effect of bacterial endotoxin on resistance of mice to ectromelia Brit J exp Path *40* 195–202, 1959

9 *Gledhill, A W* Influence of endotoxin upon the pathogenesis of viral infections In Bacterial Endotoxin Proceedings of a Symposium, Rutgers University Quinn & Boden Co Rahway, New Jersey, pp 410–421, 1964

10 *Johnson, A G* Adjuvant action of bacterial endotoxins on the primary antibody response In Bacterial Endotoxin Proceedings of a Symposium, Rutgers University, Quinn & Boden Co Rahway, New Jersey, pp 252–262, 1964

11 *Karstad, L* Aleutian disease A slowly progressive viral infection of mink In Chronic Slow Infectious Neuropathic Agents (CHINA) and Other Virus Infections. Current Topics in Microbiol and Immunol *40* 9–21, 1967

12 *Luecke, D H & Sibal, L. R* Enhancement by endotoxin of the primary antibody response to bovine serum albumin in chickens J Immun *89* 539–544, 1962

13 *Michael, J G & Massell, B F* Factors involved in the induction of non specific resistance to streptococcal infection in mice by endotoxin J exp Med *116* 101–107, 1962

14

15

role of the Granulocyte. In Bacterial Endotoxins Proceedings of a Symposium, Rutgers University, Quinn & Boden Co Rahway, New Jersey, pp 211–229, 1964

16 *Neter, E* Endotoxins and the immune response. Current Topics in Microbiol and Immunol *47* 82–124, 1969

17 *Nordstoga, K* Amyloidosis in mink induced by *repeated injections of endotoxin* Acta path microbiol scand Section A *80* 159–168, 1972

18 *Nordstoga, K & Næss, B* The effect of long-term challenge with endotoxin on the growth of Sapphire mink with high incidence of infectious plasmacytosis Acta path microbiol scand Section A *81* 344–346, 1973

19 *Porter, D D, Dixon, F J & Larsen, A E* The development of a myeloma like condition in mink with Aleutian disease Blood *25* 736–742, 1965

20 *Rahman, A N & Luttrell, C N* Pathogenesis of viral meningo-encephalitides Enhancement of viral penetrance into the brain Bull Johns Hopk. Hosp *112 1–14, 1963*

21 *Rosen, F S* The endotoxins of gram negative bacteria and host resistance New Eng J Med *264* 919–923, 1961

22 *Saison, R, Karstad, L & Pridham, T J* Viral plasmacytosis (Aleutian disease) in mink VI The development of positive Coombs tests in experimental infections Canad J comp Med *30* 151–156, 1966

Acta path microbiol scand Section A 81, 3, 352–358, 1973

MALE BREAST CANCER

3 Breast Carcinoma in Association with the Klinefelter Syndrome

OLE SCHEIKE, JAKOB VISFELDT and BENT PETERSEN

The Radium Center, Copenhagen, Institute of Pathology,
Frederiksberg Hospital, Copenhagen and Department of Radiology, Kommunehospitalet,
Copenhagen, Denmark

Thirty male patients with breast carcinoma were examined for sex chromatin One of them was found to be sex chromatin positive Chromosome analysis of peripheral blood revealed the chromosomal constitution normally found in the Klinefelter syndrome By pooling series of cases of male breast cancer, in which systematical screening for sex chromatin was made, it was found that 9 out of 242 patients were sex chromatin positive, which is a higher number than that found in the normal male population Calculated on the basis of the incidence of breast cancer in women and men, and of the incidence of sex chromatin positive individuals in the normal male population, the incidence of breast cancer among patients with the Klinefelter syndrome was found to be about one fifth of the incidence of breast cancer in women and about twenty times the incidence of breast cancer in normal men The breast carcinoma found in the sex chromatin positive patient in our material presented no special histological features Testicular histology showed a pattern different from that normally found in the Klinefelter syndrome, since no totally hyalinized a cellular tubules, which are considered pathognomonic for the Klinefelter syndrome, could be demonstrated

In 1965, *Jackson et al* (13) reported an unusually high incidence of the Klinefelter syndrome in men with breast carcinoma Among 21 male patients with breast carcinoma, three were chromatin positive and had an abnormal sex chromosome complement The incidence of breast carcinoma in the Klinefelter syndrome was calculated to be of the same order as that found in women

Since that time there has been much discussion about the possible relationship between the Klinefelter syndrome and breast carcinoma

A few case histories have later been published (6, 7, 10, 21), and series of cases of male breast cancer, including systematic screening for sex chromatin, have been reported (12, 13, 20, 23)

On the background of these data, a group of Danish patients with male breast carcinoma were screened for sex chromatin

MATERIALS AND METHODS

With the aid of the Danish Cancer Registry, 265 male patients with breast cancer were collected from the period 1 January 1943 to 1 July 1972 These patients were recorded from hospitals throughout the country, and hence the material must be considered as unselected Histological preparations from 187 cases of male breast cancer were reviewed (26) The oestradiol metabolism was studied in 19 patients (22) Buccal smears for sex chromatin determinations were obtained from 30 patients The slides were stained routinely with haematoxylin eosin, in some cases supplemented

Received 28 xii 72 Accepted 28 xii 72

Requests for reprints should be addressed to *Ole Scheike*, Radium Center, Strandboulevarden 49, 2100, Copenhagen, Denmark

by *Guard's* method for sex chromatin determination (9) At least 500 cells were screened from each patient

One of the 30 patients was found to be sex chromatin positive In this patient chromosome analysis of peripheral blood was carried out according to the method of *Moorhead et al* (19) Twenty five cells were analysed, and a large number of mitoses were counted

Testicular biopsy was planned with the object of making a histological examination, but during the operation the testes were found to be so small (greatest diameter 10 mm), that the entire right testis was extirpated The testis was fixed in Cleland's fixative Step sections were made throughout the testis. These sections were stained with haematoxylin-eosin modified van Gieson stain, and iron haematoxylin

RESULTS

The examination of buccal smears for sex chromatin of the 30 male patients with breast cancer showed 29 to be sex chromatin negative. One patient was sex chromatin positive (Fig 1) In this patient chromosome analysis on peripheral blood was made The karyotype was 47, XXY in all 25 cells analysed, i e , the normal chromosome constitution in the Klinefelter syndrome (Fig 2) In addition, a large number of mitoses were counted There was no evidence of mosaicism

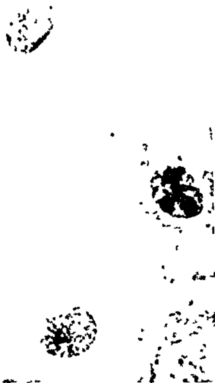


Fig 1 Buccal smear showing sex chromatin positive epithelial cells × 1200

Clinical Data

The patient was a 72 year old married man without children There was a nine months history of ulceration of the right nipple On admission an ulcerated tumour, the size of a fist was found It was fixed to the thoracic wall and replaced the right breast Because of the ulceration, palliative removal of the tumour was made Microscopic diagnosis infiltrating duct carcinoma of no special type (Fig 3) A course of radiotherapy was given to the right side of the chest, and hormone therapy was instituted (stilboestrol and prednisone) The patient deteriorated rapidly and died with lung metastases five months after establishment of the diagnosis

On admission endocrine abnormality was suspected The penis was 2 cm long and neither testis was palpable in the scrotum Growth of facial and abdominal hair and distribution of fat, however, were normal and there were no clinical signs of gynecomastia

Histology

The right testis was extirpated and a total of 60 sections were made from different levels throughout the testis

The interstitial cells The histological picture was completely dominated by Leydig cells with varying hyperplasia. The shape and size of the cells varied from one area to another In certain areas fresh blood was found in between the cells, most likely because of the operative procedure There were a number of vessels with relatively thick walls

Tubules The individual sections contained from 5-15 tubules All of these were abnormal, but uniform (Fig 4) The basement membrane was slightly thickened in some

Fig 2 Chromosomes of cells from peripheral blood with the karyotype 47, XXY

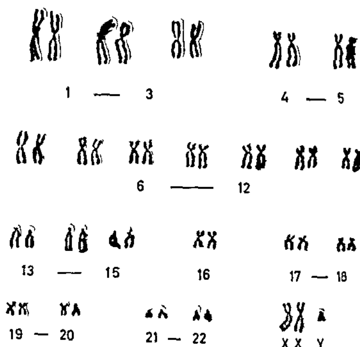




Fig 3 Infiltrating breast carcinoma of no special type × 200

areas In the lumen only Sertoli cells were found, mainly lying peripherally in a single row (Fig 5), a few cells were found in a more central position In all tubules the Sertoli cells were well differentiated with light, ovoid nuclei finely granulated chromatin and a central, distinct nucleolus Sex chromatin could not be found in the Sertoli cells In none of the tubules was spermiogenesis demonstrated Hyalinized tubules ("ghost tubules) were not seen anywhere

Hormone Assays

Before hormone therapy was started, some hormone assays were performed The urinary excretion of total gonadotrophins (determined according to the method of *Johnsen* (14, 15)) was less than 3 MUU/24 h (normal range 17-150 MUU/24 H) The excretion of total oestrogens (determined according to the method of *Brown et al* (3)) was 8 μg/24 h (normal range 8 1 23 0 μg/24 h) The excretion of 17 ketogenic steroids (determined according to the method described by *Wilson* & *Lipsett* (27)) was 8 9 mg/24 h (normal mean excretion 8 0 mg/24 h)



Fig 4 Group of preserved tubules from the testis. × 120

The 17-ketosteroids in the urine were fractionated and estimated according to the method of *Johnsen* (16). The results are shown in Table 1.

DISCUSSION

Several case histories dealing with the association between the Klinefelter syndrome and cancer of the breast have been reported In 1955, *Bauer* & *Erickson* (2) submitted the first report on this association. Since then four other case reports have been published (6, 7, 10, 21). *Keller* (18) found two cases of the Klinefelter syndrome among 181 male patients with breast cancer, but they were not documented.

In addition to these case reports, four series of cases of male breast cancer have been published, in which systematic screening for sex chromatin was made (*Schottenfeld et al.* 1963 (23), *Jackson et al.* 1965 (13), *Nadel* & *Koss* 1967 (20), *Harnden et al.* 1971 (12)).

Including the case reported in the present paper, nine documented cases of the Klinefelter syndrome were found in these series by sex chromatin screening. Hence, including the five case reports, a total of 14 documented cases of the Klinefelter syndrome occurring in association with breast cancer were reported. In all 14 cases the breast cancer was verified histologically, 10 of them in the right breast and two in the left, one bilateral, and in one case the side was not stated. This right-sided predominance, which is statistically significant $P < 0.05$), is unusual.



Fig 5 Detail of Fig. 4 Well-differentiated Sertoli cells only are seen in the tubule. × 480.

TABLE 1 *The Excretion of 17 Ketosteroids (μ mol/24h)*

	The patient	Normal Men*	
		Mean Value	Range
Androsterone	0.29	5.3	2.2–11
Etiocholanolone	0.80	4.5	1.6–11
Dehydroepiandrosterone	0.12	0.31	0.07–1.5
11 ketoandrosterone	< 0.06	0.23	< 0.03 0.69
11 ketoetiocholanolone	0.48	2.0	0.89–4.3
11 hydroxyandrosterone	< 0.37	3.3	1.6–6.2
11 hydroetiocholanolone	0.20	1.3	0.55–3.3
11 ratio §	1.0	1.6	0.65–3.4

* Men over 70 years
§ The ratio 11 desoxy 17 ketosteroids/11 oxy 17-ketosteroids

Buccal smears were obtained in 11 cases and chromosome analysis was made in 10 cases. Eight of these were of the karyotype 47, XXY, two were mosaics with 46, XY/47, XXY/48, XXXY and 46, XX/47, XXY, respectively.

Testicular biopsy was carried out in five cases.

Incidence of Breast Cancer in Patients with the Klinefelter Syndrome

In order to evaluate possible etiological factors it is essential to estimate the incidence of breast cancer in patients with the Klinefelter syndrome. To elucidate this problem the available series of cases of male breast cancer, in which systematic screening for sex chromatin was made, have been pooled (Table 2). Hereby a frequency of nine chromatin positive cases among 242 men with breast cancer was found, corresponding to an incidence of 37 per thousand. The incidence of sex chromatin positive individuals in the normal male population is stated to range from 1.9 per thousand (*Court Brown* 1969) to 2.2 per thousand (*Hambert* 1966) (5, 11).

Calculation of the incidence of breast cancer in patients with the Klinefelter syndrome involves the use of data which are somewhat inaccurate.

In Denmark today about 1600 new cases of breast cancer will be diagnosed annually in a female population of 2.5 million (4). If the average life span is estimated at 70 years, this would imply that about 112 000 out of a population of 2.5 million females would develop breast cancer or 4500 per 100 000. The incidence of male breast cancer is about 1 per cent of that of female, i.e. about 45 cases per 100 000. Hence 242 cases of male breast cancer would appear in a male population of 242/45 × 100 000, corresponding to about 500 000. Out of 500 000 men there would be about 1000 with the Klinefelter syndrome. In the pooled series nine cases were found, corresponding to an incidence of 0.9 per cent of breast cancer in patients with the Klinefelter syndrome. This is one fifth of the incidence

TABLE 2 *Chromatin Positive Males among Cases of Male Breast Carcinoma*

	No examined	No chromatin positive
Schottenfeld et al 1963	25	0
Jackson et al 1967	21	3
Nadel and Koss 1967	16	0
Harnden et al 1971	150	5
Present study	30	1
	242	9

of breast cancer in women, and 20 times the incidence of breast cancer in normal men

The validity of these calculations can be questioned because they are based on the following assumptions a stationary population (average age being applied) a uniform age distribution for cases of breast cancer in women men, and patients with the Klinefelter syndrome, and a random selection of individuals examined from the cases listed in Table 2 (the lethality in the various age groups exerts an influence in this connection) Therefore the figures obtained can be considered only as a first approximation in the assessment of the order of magnitude of the incidence of breast cancer among patients with the Klinefelter syndrome

On the basis of their own group of patients *Jackson et al* (13) (cf Table 2) calculated the incidence of breast cancer in the Klinefelter syndrome to be of the same order as the incidence in women On the basis of more recent series it seems as stated above, that the incidence of breast cancer in patients with the Klinefelter syndrome increased heavily, as compared with the normal male population Nevertheless the incidence is considerably lower than that found in women

Hormonal Aspects

Of possible causes for the increased frequency of breast cancer in Klinefelter patients the hormonal imbalance and the frequently occurring gynecomastia should be mentioned

In the Klinefelter syndrome the gonadotrophin excretion is characteristically increased (25) In our patient the excretion of pituitary gonadotrophin was below normal Measurements of the gonadotrophin excretion were also made in five of the published cases of the Klinefelter syndrome in association with breast cancer (6 13 21) Three of the five patients had high excretion levels

The excretion of total 17 ketosteroids ranges between low and normal in patients with the Klinefelter syndrome but can hardly be considered relevant in the assessment of the testicular function However it was measured in six of the cases reported (6, 7, 13 21), and in two cases it was stated to be low

The fractionated 17 ketosteroids are of greater interest In our patient (Table 1), the values were found to be low for androsterone and etiocholanolone and also an 11-ratio in the low range was found A low ratio of 11 desoxy 17 ketosteroids/11-oxy 17 ketosteroids (11 ratio) is a sign of hypogonadism (*Johnsen* (16))

The excretion of total oestrogens is normal or decreased in patients with the Klinefelter syndrome (25 8) In our patient this value was within normal limits

The relationship between gynecomastia and breast cancer is much discussed In patients with the Klinefelter syndrome, gynecomastia occurs frequently but it was not present in our patient In only four of the previously published 13 cases of the Klinefelter syndrome associated with breast cancer, was gynecomastia found

Testicular Histology

The testicular histological picture in patients with the Klinefelter syndrome is first and foremost characterized by totally hyalinized a cellular tubules This finding is considered to be pathognomonic for the Klinefelter syndrome (17) The non hyalinized tubules contain in most cases Sertoli cells only however there is occasionally some spermiogenesis in individual tubules in the testicular biopsy specimens (e g (24)) In addition there is proliferation of the Leydig cells often resembling nodular hyperplasia

In our patient no hyalinized tubules were found This is apparently exceptional and we are unable to provide any explanation The few tubules present all contained Sertoli cells only mainly arranged peripherally in a single row The Sertoli cells were well differentiated –cf the description Hence in our patient we found tubules of type A only (*Skakkebæk* (24)) Furthermore the tubules were often found in relation to the dense Leydig cell aggregates As discussed inter alia by *Ahmad* (1) it may be that the Leydig cell hyperplasia is only apparent and that the total number

of Leydig cells in the Klinefelter syndrome is normal

Our thanks are due to Mr *Arne Nielsen*, Cand Act, University Lecturer, for his critical review of the statistical calculations

Surgical Department I and Medical Department II, Kommunehospitalet, Copenhagen are thanked for valuable help

The study was supported by a grant from the *National Anti Cancer League*

REFERENCES

1 *Ahmad, K A, Dykes, J R W, Ferguson Smith, M A, Lennox, B & Mack, W S* Leydig cell volume in chromatin positive Klinefelter s syndrome J clin Endocr *33* 517–520 1971

2 *Bauer, D F & Erickson, R L* Male breast cancer Klinefelter syndrome with prostatic, adrenal and mammary tumors Northw Med *54* 472–476,1955

3 *Brown, J B, Mac Leod S C, Mac Naughtan C Smith, M A & Smyth, B* A rapid method for estimating oestrogens in urine using a semi automatic extractor J Endocr *42* 5–15 1968

4 *Clemmesen, J* Statistical studies in the aetiology of malignant neoplasms I, II and III Acta path microbiol scand Suppl 1965 and 1969

5 *Court Brown W M* Sex chromosome aneuploidy in man and its frequency with special reference to mental subnormality and criminal behaviour Int Rev exp Path *7* 31 97 1969

6 *Cuenca C R & Becker, K L* Klinefelter s syndrome and cancer of the breast Arch intern Med *121* 159 162 1968

7 *Dodge O G, Jackson, A W & Muldal, S* Breast cancer and interstitial cell tumor in a patient with Klinefelter s syndrome Cancer *24* 1027 1032 1969

8 *Giorgi E P & Sommerville, I F* Hormone assays in Klinefelter s syndrome J clin Endocr *23* 197 202 1963

9 *Guard H R* A new technic for differential staining of the sex chromatin and the determination of its incidence in exfoliated vaginal epithelial cells Amer J clin Path *32* 145 151, 1959

10 *Gupta, R L* Carcinoma of the breast in a case of Klinefelter syndrome Indian J Cancer *3* 184–189 1966

11 *Hambert, G* Males with positive sex chromatin Thesis Akademiforlaget Gothenburg *1966*

12 *Harnden, D G, Maclean N & Langlands, A O* Carcinoma of the breast and Klinefelter s syndrome J med Genet *8* 460-461 1971

13 *Jackson, A W, Muldal, S, Ockey, C H & O Connor, P J* Carcinoma of male breast in association with the Klinefelter syndrome Brit med J *1* 223–225, 1965

14 *Johnsen, S G* A clinical routine method for the quantitive determination of gonadotrophins in 24 hour urine samples Acta endocr (Kbh) *28* 69 88, 1958

15 *Johnsen, S G* A clinical routine method for the quantitive determination of gonadotrophins in 24 hour urine samples II Normal values for men and women at all age groups from pre puberty to senescence Acta endocr (Kbh) *31* 209–227, 1959

16 *Johnsen, S G* To be published

17 *Johnsen, S G* Testicular biopsy score count A method for registration of spermatogenesis in human testes normal values and results in 335 hypogonadal males Hormones *1* 1 24 1970

18 *Keller, A Z* Demographic clinical and survivorship characteristics of males with primary cancer of the breast Amer J Epidem ol *85* 183–199 1967

19 *Moorhead, P S, Nowell P C, Mellman W J, Battips, D M & Hungerford D A* Chromosome preparations of leucocytes cultured from human peripheral blood Exp Cell Res *20* 613 616, 1960

20 *Nadel, M & Koss L G* Klinefelter s syndrome and male breast cancer Lancet 2 366 1967

21 *Robson M C, Santiago Q & Huang T W* Bilateral carcinoma of the breast in a patient with Klinefelter s syndrome J clin Endocr *28* 897 902 1968

22 *Scheike O, Svenstrup B & Frandsen, V A* Male breast cancer 2 Metabolism of oestradiol 17β in men with breast cancer To be published

23 *Schottenfeld D Lilienfeld A M & Diamond H* Some observations on the epidemiology of breast cancer among males Amer J publ Hlth *53 890–897 1963*

24 *Skakkebæk N E* Two types of tubules containing only sertoli cells in adults with Klinefelter s syndrome Nature (Lond) *223* 643 645 1969

25 *Stewart J S S Mack W S Govan, A D T Ferguson-Smith M A & Lennox B* Klinefelter s syndrome Clinical and hormonal aspects Quart J Med *28* 561 571, 1959

26 *Visfeldt J & Scheike O* Male breast cancer 1 Histological typing and grading of 187 Danish cases To be published

27 *Wilson H & Lipsett M B* Use of periodate oxidation in the clinical analysis of urine corticoids Analyt Biochem *5* 217 225 1963

Acta path microbiol scand Section A 81, 3, 359-365, 1973

MALE BREAST CANCER

4 Gynecomastia in Patients with Breast Cancer

OLE SCHEIKE and JAKOB VISFELDT

The Radium Center, Copenhagen and Institute of Pathology, Frederiksberg Hospital
Copenhagen, Denmark

In order to elucidate the question of whether or not gynecomastia is a premalignant state, a Danish series comprising 265 cases of male breast cancer was reviewed with regard to a previous history of gynecomastia and the finding of clinical gynecomastia on admission Furthermore, preparations from 187 cases of the same series were assessed with a view to the presence of histologically verified gynecomastia. In 10 patients there was a history of gynecomastia, only one patient presented clinical gynecomastia on admission In 79 cases there was sufficient breast tissue for a histological study, this revealed gynecomastia in 21 cases, 6 of which were of florid type (type I) and 15 of quiescent fibrous type (type II) In 2 cases ducts with severe atypia of the epithelium were found, but in spite of investigations of serial sections no transition into invasive growth was revealed It is concluded that the following features seem to support the theory that gynecomastia may be a premalignant state A the finding of severe atypia of the epithelium in the ducts in concurrent gynecomastia and breast cancer, B lower mean age in cases of breast cancer with concurrent gynecomastia, C. the higher ratio of male to female breast cancer and the low mean age of the male patients in an area with a high frequency of gynecomastia, D heavily increased frequency of breast cancer in patients with the Klinefelter syndrome

The possible association between gynecomastia and breast cancer has been discussed in almost all reports on series of male breast cancer, and several series give information about anamnestic objective and histological gynecomastia Hence, *Norris* & *Taylor* found gynecomastia in 5 per cent of 108 patients (11) Out of the 40 patients reported on by *Liechty* (7), 7 had gynecomastia *Sinner* (15) found 5 cases in 27 patients. *Huggins* & *Taylor* (4) reported only one case of gynecomastia among 75 patients, and *Holleb et al* (3) found only 2 cases among 198 patients

In order to elucidate the question of whether or not gynecomastia is a premalignant state, a comprehensive Danish series of cases of breast cancer was reviewed with regard to the presence of gynecomastia

Received 6 1 73 Accepted 6 1 73

Requests for reprints should be addressed to *Ole Scheike*, Radium Center, Strandboulevarden 49, 2100, Copenhagen, Denmark

MATERIALS AND METHODS

With the aid of the Danish Cancer Registry, 265 male patients with breast cancer were traced for the period 1 January 1943 to 1 July 1972 from all over Denmark Histological preparations from 187 cases of male breast cancer were re assessed (17) Oestradiol metabolism was investigated in 19 patients (13)

In connection with the clinical and histological review of these 265 cases examination for the presence of gynecomastia was made This consisted in a survey of a) anamnestic gynecomastia, b) clinical gynecomastia on admission and c) histologically verified gynecomastia based on preparations from the 187 cases of male breast cancer

RESULTS

Anamnestic Gynecomastia

In 10 patients there was a history of homolateral gynecomastia In 5 of the cases the condition was bilateral Three of the 10 patients had gynecomastia during puberty and in two of the cases it was irreversible Two patients had had reversible gynecomastia during adult life In the remaining 5 patients, who all had had gynecomastia as adults, operation or biopsy had been carried out from 5½ to 16 years prior to establishment of the cancer diagnosis In four of the cases the histological preparations were available and were reviewed The histological diagnoses were mastitis, gynecomastia type I, gynecomastia type I with mastitis, gynecomastia type I with pronounced proliferation of the epithelium

Clinical Gynecomastia on Admission

Only one patient had palpable gynecomastia on admission This was bilateral gynecomastia associated with clinically verified cancer in the left breast The patient is also registered under "anamnestic gynecomastia"

Histological Gynecomastia

In 79 of the 187 cases there was sufficient breast tissue for a more exact histological evaluation In 21 of these 79 cases, there were histological signs of gynecomastia, i e, 6 of type I and 15 of type II, cf the discussion

DISCUSSION

Anamnestic Gynecomastia

According to the case reports, 10 of our patients presented a history of gynecomastia, of these 3 had gynecomastia during puberty

In many studies on male breast cancer, anamnestic gynecomastia is included among the number of cases of gynecomastia Apart from the usual inaccuracy pertaining to anamnestic data, a history of gynecomastia is only slightly relevant in the assessment of the condition as a possible premalignant state, gyncomastia being fairly frequent in the normal population Hence, *Nydick* (12) found clinical gynecomastia in 65 per cent of normal boys of the age of 14 years Gynecomastia among adults is far from rare, the maximum frequency occuring at the age of 50 years (16) -(cf Fig 7)

Clinical Gynecomastia on Admission

According to the case records, only one of our patients had clinical gynecomastia on admission Histologically verified gynecomastia is, however, not always associated with enlarged breasts Hence, *Williams* (18) found enlargement of the breasts in only 4 out of 178 cases with histological gynecomastia

Histological Gynecomastia

The histological criteria for gynecomastia vary somewhat from one author to another *Williams* (18) used two types of gynecomastia, types I and II, and in the present study this classification has been adopted

Type I (florid type), Figs 1, 2, is characterized by an increased number of ducts with irregular lumen, in some cases showing pseudolobule formation (Fig 5) Proliferation of the epithelium is present, defined as three or more layers of cells, sometimes with budding or small papillae Around the ducts cuffs are found, consisting of characteristic, light, very loose connective tissue stroma, usually containing a few round cells, in some cases eosinophilic granulocytes. These cuffs are fairly well demarcated from the 'interlobular' connective tissue This is increased in volume and consists of dense collagen tissue with varying fibroblastic proliferation

Type II (quiescent fibrous type), Figs 3, 4 shows a slightly increased number of ducts

Figs 1 2 Gynecomastia type I florid type *Ducts* with irregular outline epithelial proliferation and periductal cuffs of loose connective tissue × 120

Figs 3–4 Gynecomastia Type II quiescent, fibrous type Duct with ectasia and irregular outline No epithelial proliferation no cuffs Dense, fibrous interlobular stroma × 30


3
4

TABLE 1 *Histological Findings in 21 Cases of Gynecomastia Associated with Mammary Cancer*

Age	Ductal proliferation	Irregular outline of ducts	Epithelial proliferation	Budding	Cuffs	Periductal round cell infiltration	Interlobular stroma fibrosis	Interlobular stroma hyalinization	Apocrine glands	Type
71	+	+	+	+	+	+	+		+	I
63	+	+	+		+	+	+		+	I
59	+	+					+	+		II
50	+	+					+	+		II
39	+	+					+			II
46	+	+	+		+	+	+			I
63	+	+					+			II
56	+	+					+	+		II
63	+	+					+			II
58	+	+	+		+	+	+			I
68	+	+	+	+			+	+		II
72	+	+	+	+		+	+	+		II
40	+	+				+	+	+		II
36	+	+					+	+		II
49	+	+					+	+		II
56	+	+	+	+	+	+	+	(+)		I
47	+	+					+	+		II
59	+	+	+	+	+	+	(+)	+		I
80	+	+			+	+	+	+		II
48	+	+					+	+		II
41	+	+		(+)			+	+	+	II

with irregular lumen and often slight ectasia Little or no proliferation of the epithelium is present, and seldom budding and small papillae Normally no cuffs are seen, the interlobular tissue extends as far as to the basement membrane The stroma is increased in volume, often with hyalinization and without pronounced fibroblastic proliferation

The most important histological findings in the 21 cases of gynecomastia in our series are shown in Table 1

Ductal proliferation is difficult to assess There is both a qualitative element (pseudolobule formation) and a quantitative element A reliable definition of the number of lobules must require examination of the entire breast, and this we were unable to do However, we estimated that there was an increased number of ducts in all cases, but often to a modest extent in type II Cuffs of loose connective tissue is the predominant feature in type I, and it was registered in all cases of type I and in one case of type II Proliferation of the epithelium is characteristic primarily in type I, but may occur in type II An increased quantity of interlobular stroma is found in all cases, hyalinization was present mainly in type II, although, in a single case, we observed a small hyalinized area in a type I Apocrine glands are said to be a fairly frequent finding in gynecomastia (18), we found this in a total of three cases

The table and the above comments show that we are unable to establish any definite criteria for a differentiation between types I and II The histological characteristics of the two types overlap to a certain extent, which also appears from the report by *Nicols et al* (10) Consequently the classification into types I and II is based on a collective evaluation of the histological findings

Our cases are distributed into 6 (29 per cent) of type I and 15 (71 per cent) of type II This corresponds fairly well to the distribution in *Williams'* series (18) which included 21 per cent of type I and 79 per cent of type II out of 178 autopsy cases with histo-





Fig 5 Gynecomastia type I Pseudolobule formation. × 30

Fig 6 Gynecomastia type I Severe atypia of ductal epithelium × 480

logical gynecomastia As regards the frequency of histological gynecomastia there was sufficient breast tissue for evaluation in only 79 cases of the 187 cases of male breast cancer which form our basic material We found histological gynecomastia in 21 of these cases corresponding to 27 per cent By examining breast tissue from 447 consecutive autopsy cases *Williams* found histological gynecomastia in 178 cases corresponding to 40 per cent The difference in the incidence of histological gynecomastia in the two series may perhaps be explained by the more limited quantity of breast tissue available in our sections

More recent studies (10) indicate that there is a relationship between the histological type of gynecomastia and the duration of the condition Hence it was found that the florid type I occurs most frequently among fresh cases and that type II the fibrous type, is most frequent in cases with a long history of the disease (10)

Our series included two cases of gynecomastia type II in which histological preparations of tissue taken 16 years and 6 years previously showed gynecomastia of type I Although there are only two cases these seems to substantiate the theory of a relationship between the histological type and the duration of the condition

Histological Gynecomastia and Focal Beginning of Cancer

The transition from gynecomastia to carcinoma is thought by several authors (2, 6, 8) to have been proved histologically However this demonstration is extremely difficult, if the criterion is invasive growth of epithelium

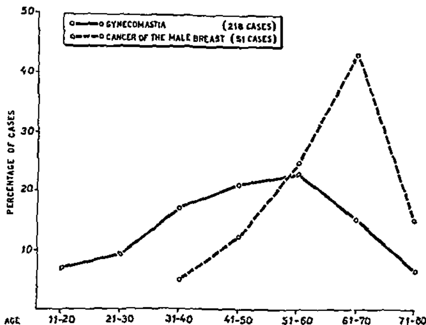


Fig 7 Age distribution of males with gynecomastia and breast cancer Reproduced by permission of *Professor Carlo Sirtori, Milan,* and *Cancer* (16)

from the ducts into areas with gynecomastia The probability of a transition can be supported by demonstration of ducts with epithelium showing carcinoma in situ (9) In two of our cases we found ducts with abnormal epithelium in the gynecomastia co-existing with breast cancer (Fig 6) In spite of examination of serial sections, we were unable to demonstrate direct transition into invasive growth

Senescent Gynecomastia in Male Breast Cancer

It is well known that gynecomastia has two incidence maxima one in puberty, and the other between the ages of 50 60 years (12, 16) *Sirtori* found a maximum incidence of gynecomastia at the age of 55 and a maximum incidence of breast cancer at 65 years of age (cf Fig 7) In our series of male breast cancer comprising 265 cases the average age was 64 8 years In the 21 cases with concurrent histological gynecomastia, the average age was 55 4 years, which is significantly lower ($P < 0\,01$)

In Egypt, a remarkably high ratio of male to female breast cancer of about 1 to 15 was found (1) At the same time there is a high incidence of gynecomastia The average age for the diagnosis of male breast cancer in that country is 41 years Hence, the same tendency seems to apply as that found in our series early development of breast cancer in association with gynecomastia, indicating a possible etiological relationship between gynecomastia and breast cancer

Gynecomastia and Breast Cancer in Cases of the Klinefelter Syndrome

Gynecomastia is a cardinal symptom in the Klinefelter syndrome (5) On the basis of literary studies and our own series, we found an incidence of breast cancer in the Klinefelter syndrome of about 20 times the incidence of breast cancer in normal males (14) This is a further indication of a possible etiological relationship between gynecomastia and breast cancer

CONCLUSION

It must be concluded that none of the features reviewed would prove the existence of an

etiological relationship between gynecomastia and breast cancer

However, certain facts appear to support the theory that gynecomastia may be a premalignant state Among these the following should be emphasized A the finding of severe atypia of ductal epithelium in certain cases with concurrent gynecomastia and breast cancer, B the lower mean age in cases of breast cancer with concurrent gynecomastia, C the higher ratio of male to female breast cancer and the low mean age of the male patients in an area with a high frequency of gynecomastia, D the heavily increased frequency of breast cancer in patients with the Klinefelter syndrome, where gynecomastia is a cardinal symptom

---

The work was supported by a grant from the *National Anti Cancer League*

REFERENCES

1 *El Gazayerli, M M & Abdel Aziz, A S* On bilharziasis and male breast cancer in Egypt a preliminary report and review of the literature Brit J Cancer *17* 566–571, 1963

2 *Gleichmann, H G* Die beziehungen zwischen gynakomastie und karzinom der mamma Z ges inn Med *8* 567 570, 1953

3 *Holleb, A I, Freeman, H P & Farrow, J H* Cancer of the male breast, part I N Y St J Med *68* 544–553, 1968

4 *Huggins, C & Taylor, G W* Carcinoma of male breast Arch Surg *70* 303–308 1955

5 *Klinefelter, H F, Reifenstein, E C & Albright, F* Syndrome characterized by gynecomastia, aspermatogenesis without A Leydigism and increased excretion of follicle stimulating hormone J clin Endocr 2 615–627, 1942

6 *Krückemeyer, K* Dysplasien des drusenepithels bei fibrosis mammae virilis Beziehungen zum karzinom Munch med Wschr *110* 2798–2801, 1968

7 *Liechty, R D, Davis, J & Gleysteen, J* Cancer of the male breast Cancer *20* 1617 1624, 1967

8 *Lyall, A* Chorioncarcinoma of the testis with gynecomastia. Report of a case with early breast cancer Brit J Surg *34* 278–280 1947

9 *McDivitt, R W, Stewart, F W & Berg, J W* Tumors of the breast Atlas of Tumor Pathology Second series. Armed Forces Institute of Pathology, Washington D C, 1968, p 106–107

10 *Nicolis G L, Modlinger, R S & Gabrilove, J L* A study of the histopathology of human gynecomastia J clin Endocr *32* 173–178, 1971

11 *Norris, H J & Taylor, H B* Carcinoma of the male breast Cancer *23* 1428–1435, 1969

12 *Nydick, M, Bustos, J, Dale, J H & Rawson, R W* Gynecomastia in adolescent boys J Amer med Ass. *178* 449–454, 1961

13 *Scheike, O Svenstrup, B & Frandsen, V A* Male breast cancer 2 Metabolism of oestradiol 17 β in men with breast cancer To be published

14 *Scheike, O, Visfeldt, J & Petersen, B* Male breast cancer 3 Breast carcinoma in association with the Klinefelter syndrome Acta path microbiol scand Sect A *81* 352–358, 1973

15 *Sinner, W* Karzinome der mannlichen brustdrüse Beobachtungen an 27 fallen. Zurcher erfahrungen 1919 bis 1960 Strahlentherapie *115* 522 547, 1961

16 *Sirtori, C & Veronesi, U* Gynecomastia A review of 218 cases Cancer *10* 645–654, 1957

17 *Visfeldt, J & Scheike, O* Male breast cancer 1 Histological typing and grading of 187 Danish cases To be published

18 *Williams, M J* Gynecomastia Its incidence, recognition and host characterization in 447 autopsy cases. Amer J Med *34* 103–112, 1963

Acta path microbiol scand Section A *81, 3*, 366–375, 1973

THE SYMPATHETIC MYOCARDIAL INNERVATION IN VARIOUS FORMS OF EXPERIMENTAL CARDIAC HYPERTROPHY

A Histochemical and Ultrastructural Study

GUNNAR UNGE, EUGEN MANDACHE* and ARNE LJUNGQVIST

The Departments of Pathology and Thoracic Medicine, Karolinska sjukhuset, Stockholm, Sweden

The sympathetic innervation of the hypertrophied myocardium was investigated by the histochemical fluorescence method for biogenic monoamines and electron microscopy Cardiac hypertrophy was induced by renal hypertension, aortic stenosis and swimming exercise. An increase in the density of adrenergic structures was observed in relation to small vessels in the left cardiac wall in swimming exercised rats and, although to a much less extent in the hypertensive rats In relation to the larger myocardial vessels an increased density was observed in swimming exercised rats and in rats with long term hypertension In the electron microscope, nerve structures were seen adjacent to growing capillaries The number of nerve fibres and filaments related to each vessel was not increased No change was observed in the rats with aortic stenosis The observations indicate that in cardiac hypertrophy secondary to swimming exercise the newly formed myocardial blood vessels aquire a normal adrenergic nerve supply

The adrenergic nervous system has a perivascular distribution in the normal heart (*Angelakos et al* 1963, *Dahlström et al* 1965, *Ehinger et al* 1966, *Jacobowitz et al* 1967, *Krokhina* 1969 and *Winckler* 1969) This pattern of innervation has been reported also in the hypertrophied heart (*Winckler* 1969 and *Vogel et al* 1969), but no comparative studies of the myocardial adrenergic nerve patterns in cardiac hypertrophy of various origins have been presented In a series of recent studies we have demonstrated an unquestionably significant neoformation of myocardial blood vessels in the enlarging hearts of rats subjected to swimming exercise, but not in the hypertrophied hearts from rats with arterial hypertension and aortic stenosis (*Ljungqvist & Unge* 1972 *Mandache et al* 1972, *Ljungqvist & Unge* (13) and *Mandache et al* 1973) In the present investigation these three forms of cardiac hypertrophy were compared with respect to the sympathetic myocardial innervation, as revealed by the histochemical fluorescence method for biogenic monoamines and electron microscopy

Received 24 xi 72 Accepted 17 i 73

* WHO fellowship No 70T ROM 0008

Requests for reprints should be addressed to *Arne Ljungqvist*, M D Department of Pathology Karolinska sjukhuset, S 104 01 Stockholm, Sweden

MATERIAL AND METHODS

Female Sprague Dawley rats were used their initial weights ranging between 190 and 200 g The animals were housed in cages, 3–4 rats in each cage

and were fed a standard laboratory diet containing 0.4 per cent sodium chloride, and tap water *ad libitum*

Cardiac hypertrophy was induced through (i) renal hypertension by clipping the left renal artery, (ii) aortic stenosis and (iii) swimming exercise (*Ljungqvist & Unge* 1972) The rats were grouped as follows

Group I A	Twenty rats which were killed during the first week of hypertension
Group I B	Eleven rats which were killed after 3 months of hypertension
Group II	Nineteen rats which were killed 2 months after the production of aortic stenosis
Group III A	Twelve rats which were killed after 3 months of swimming exercise
Group III B	Twelve rats which were killed after 3 months of swimming exercise followed by a resting period of 2 months
Group IV A	Twelve normal rats the age and body weight of which made them comparable to the rats of groups II and III A
Group IV B	Twelve normal rats the age and body weight of which made them comparable to the rats of groups I B and III B
Group IV C	Nine rats in which the production of left renal artery stenosis was not followed by hypertension These rats were compared with the rats of group I A

At the end of the experimental periods the heart in one rat from each group was fixed by perfusion of a 1.5 per cent glutaraldehyde solution *via* a catheter inserted in retrograde direction into the abdominal aorta of the anaesthetized animal The animals died during the perfusion Pieces were taken from the left ventricle wall of these hearts for electron microscopy

All the other animals were killed by an overdosage of ether and their hearts were quickly removed and weighed From the left ventricle wall of each of these hearts small pieces were fixed for electron microscopy by immersion in 4 per cent glutaraldehyde buffered to pH 7.4 The remainder of the heart was processed for histochemical demonstration of biogenic monoamines (*Falck et al* 1962)

Histochemical Fluorescence Method

[illegible]

The rest of the heart was immersed in an ice cold modified Krebs Ringer bicarbonate buffer solution for a minimum period of 5 minutes after which it was removed and placed on a cold surface The heart was held in position by compressing it from above with an object glass Thin slices were prepared by sliding a razor blade along the lower surface of the object glass These slices were then incubated during continuous shaking in the buffer solution alone and in buffer solution containing α methyl norepinephrine Two different concentrations of α methyl norepinephrine were used, 5×10^{-4}M and 5×10^{-5}M After 30 min, the slices were washed with the buffer solution for 10 min (see *Hamberger* 1967) followed by freeze drying at −30°C

After the freeze drying procedure, both the incubated and non incubated slices were exposed to formaldehyde gas (water content of formaldehyde powder 0.6 per cent) for 1 hour at +80°C and vacuum-embedded in paraffin 2–3 μ thick sections were cut from the blocks and mounted in Entellan (Merck) to which Xylol was added The sections were examined in the fluorescence microscope (For further details on the histochemical technique see *Fuxe et al* 1970 and *Olson & Ungerstedt* 1970)

The microscopical examination was performed twice with an interval of several weeks, fresh sections being examined each time At the examination the density of fluorescent varicose fibres and their fluorescence intensity were evaluated according to a scale ranging from + (normal) to +++ (heavily increased) The material was coded to make the examiner ignorant of the clinical history

Electron Microscopy

All the glutaraldehyde fixed material was postfixed in OsO_4 embedded in Epon, sectioned in an Ultrotome (LKB) and studied in a Siemens Elmiskop I For a quantitative evaluation of the innervation at the capillary level, the number of axons and filaments related to 100 capillaries in a section [illegible] in group I B, such determination could not be performed due to technically unsatisfactory material

RESULTS

In Table 1, the heart/body weight ratios of the different groups of animals are presented, for practical reasons multiplied by 1000 A significantly increased ratio, indicative of cardiac hypertrophy, was recorded in all experimental animals, with the exception of the groups of animals whose swimming exercise was followed by a period of rest, and the normotensive animals with left renal artery stenosis

TABLE 1 *Effect of Renal Hypertension, Aortic Stenosis and Swimming Exercise on the Heart Weight (Ratio) of Rats, on the Density of Varicose Adrenergic Fibres in the Myocardium and on the Number of Axons and Nerve Filament' per 100 Myocardial Capillaries*

Group	Ratio	Varicosity density		Axons	Filaments
		large vessels	small vessels		
I A	4 4 ± 0 6§	+	+ +	65 ± 10	160 ± 15
I B	4 8 ± 1 0§	+ +	+ +	–	–
II	4 1 ± 0 4*	+	+	62 ± 5	168 ± 8
III A	4 4 ± 0 3†	+ +	+ + +	70 ± 8	–
III B	3 8 ± 0 3	+	+	68 ± 5	170 ± 6
IV A	3 8 ± 0 3	+	+	65 ± 11	165 ± 10
IV B	3 7 ± 0 5	+	+	68 ± 4	168 ± 11
IV C	3 7 ± 0 4	+	+	–	–

Group I A renal hypertension 1 week Group I B renal hypertension 3 months Group II aortic stenosis 2 months Group III A swimming exercise 3 months Group III B swimming exercise, 3 months, followed by rest, 2 months Group IV A–C control rats Ratio heart × 1000/body weight The figures are means ± SD + = normal density, + + = increased density, + + + = heavily increased density

* Significantly different from control group (0 01 < p < 0 05)

§ Significantly different from control group (0 001 < p < 0 01)

† Significantly different from control group (p < 0 001)

Fig 1 Fluorescence microphotograph of left ventricle wall of normal rat heart showing longitudinally sectioned nerves which appear as varicose fibres in the interstitial tissue × 800

Histochemical Fluorescence Method

In the fluorescence microscope the adrenergic nerves appeared as varicose fibres with a yellowish green fluorescence if oriented parallel to the plane of section (Fig 1) and as fluorescent dots if oriented perpendicularly to this plane (Fig 3) The fibres were located in the interstitial tissue both around wide vessels of various calibres (Fig 2 A) and between the muscle cells where they might well have had a pericapillary distribution (Figs 1 and 3 A) The material incubated with norepinephrine of both concentrations did not show any deviations from the pictures seen in the non-incubated material, with the possible exception of a slight degree of diffusion after incubation

Both in the incubated and the non incubated material an increased density of fluorescent varicose fibres in the interstitial areas, probably in relation to capillaries, was recorded in the left ventricle wall of swimming exercised rats that had not rested (Fig 3 B), and to a certain degree in the hypertensive rats (Table 1) In the hypertrophied hearts from swimming exercised rats and rats with long-term hypertension the density of fluorescent varicose fibres appeared to be increased also



Fig 2 *a* Fluorescence microphotograph of left ventricle wall of normal rat heart showing normal numbers of varicose fibres around an artery × 800

Fig 2 *b* Fluorescence microphotograph of left ventricle wall of hypertrophied rat heart (hypertension 3 months) showing increased numbers of varicose fibres around an artery giving the impression of an increase in the fluorescence intensity × 800

a

b

Fig 3 *a* Fluorescence microphotograph of left ventricle wall of normal rat heart showing cross-sectioned nerves which appear as fluorescent dots in the interstitial tissue × 600

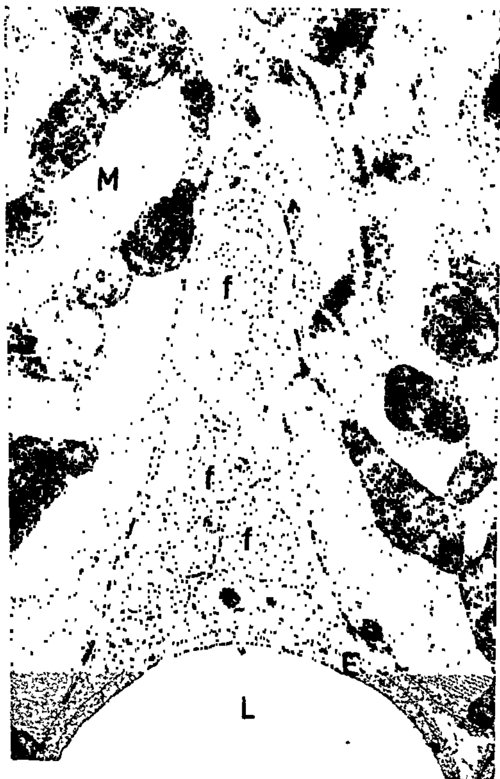
Fig 3 *b* Fluorescence microphotograph of left ventricle wall of hypertrophied rat heart (swimming exercise 3 months) showing increased numbers of cross sectioned nerves in the interstitial tissue × 600



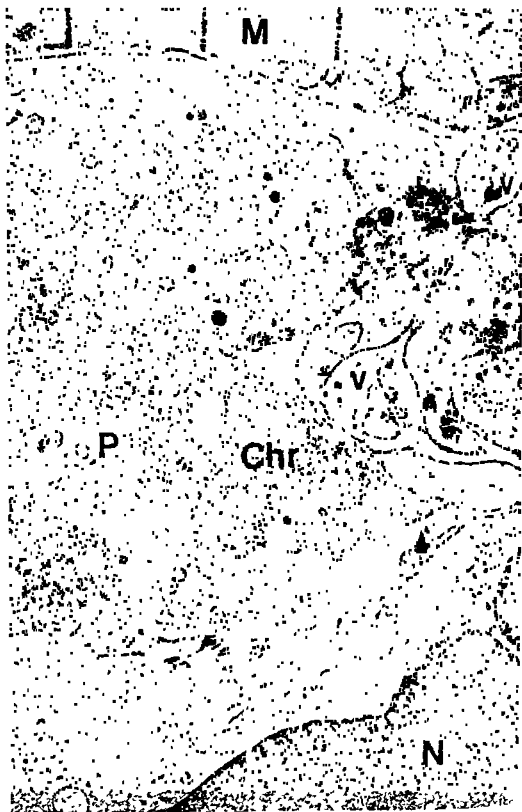
Fig 4 Cross section of myocardium from normal rat showing vegetative nerve structure containing filaments (f) and a varicosity (V) Part of fibroblast (Fb) Lumen of blood capillary (L) Cardiac muscle cell (M) × 22 400

around the larger myocardial arteries in the left ventricle wall (Table 1), thus giving the impression of an increased fluorescence intensity around these arteries (Fig 2 B) No clear differences, however, in the intensity of the fluorescence reaction indicative of different levels of norepinephrine (*Olson et al* 1968)

Fig 5 Cross section of myocardium *from rat* after 3 months' swimming exercise showing *numerous* nervous filaments (f) close to a *blood capillary* lumen (L) Endothelial wall (L) *Cardiac* muscle cell (M) × 16 800



24*


M
V
V
P
Chr
N

in the various groups of rats were recorded In rats in which swimming exercise was followed by a period of rest and in rats with aortic stenosis, no nerve changes were observed in the fluorescence microscope Nor were any nerve changes observed in the right ventricle wall in any of the groups

Electron Microscopy

In the electron microscopical investigation, interest was focused on the nerve structures at the capillary level Here nerve fibres, Schwann cells and varicosities were found in all groups of rats The axons varied in width and most often consisted of 2–4 filaments (Fig 4), but in occasional areas the filaments were more numerous (Fig 5) There were no significant differences between the rats of the various groups with respect to their numbers of myocardial axons and nerve filaments per 100 capillaries (Table 1) In the swimming exercised rats, ultrastructural features of capillary neoformation were present (*Mandache et al* 1972) and thin nerve filaments and varicosities were seen adjacent to these growing capillaries (Fig 6)

DISCUSSION

Studies of the normal heart by the histochemical fluorescence method have shown a perivascular distribution of the adrenergic nervous system (*Angelakos et al* 1963, *Dahlström et al* 1965, *Ehinger et al* 1966, *Jacobowitz et al* 1967, *Krokhina* 1969 and *Winckler* 1969) In cardiac hypertrophy induced by pulmonary stenosis, *Vogel* (1969) found a decreased density of the myocardial adrenergic nerves, whereas no nerve alterations were found by *Winckler* (1969) in hearts from rats subjected to swimming exercise for 1 hour These findings suggest different reactions of the myocardial adrenergic nervous system in cardiac hypertrophy of different origins, an assumption which gains further support by the present observations Thus, if applied to the present material, the histochemical fluorescence method revealed an increased density of adrenergic nerves in the left ventricle wall of hearts from rats in which cardiac hypertrophy was induced by swimming exercise A much smaller increase in density was seen in the left ventricle walls from hypertensive rats No nerve changes were observed in the hearts from rats with aortic stenosis and rats whose swimming exercise was followed by rest

The observed nerve reactions seem to parallel the previously reported reactions in the finer myocardial vasculature in cardiac hypertrophy of different origins (*Ljungqvist & Unge* 1972), suggesting that the neoformation of myocardial blood vessels in the exercising rat (*Mandache et al* 1972 and *Mandache et al* 1973) is associated with the development of a perivascular nervous system of the growing vessels This was further evidenced by the electron microscopical observations and in full agreement with the increase in the norepinephrine content per g heart tissue recorded by *Östman et al* (1972) in exercised rats In hypertensive rats, only vague features of myocardial capillary neoformation have been noted (*Ljungqvist & Unge* 1972, *Ljungqvist & Unge* (13)) and the increased density of adrenergic structures was slight in these rats It can be anticipated that the development of an apparently normal adrenergic innervation of the newly formed myocardial vessels in the exercised rats is a necessary prerequisite for the adequate function of these vessels

Previous observations have suggested that the adrenergic nerves of an organ may be-

method (*Dahlström & Zetterström* 1965 *Ljungqvist & Ungerstedt* 1972) This, of course, will give a false impression of the density of nerves in the organ In order to as-

Fig 6 Section of myocardium from rat after 3 months swimming exercise showing interstitial area with part of a dividing pericyte (P) close to nerve endings including varicosities (V) Chromosomes (Chr) Endothelial cell nucleus (N) Cardiac muscle cell (M) × 16 800

certain whether any such reaction would explain the observed differences between the various groups of rats, portions of the hearts from each group were incubated with α-methyl-norepinephrine before reacting with formaldehyde gas, this can be expected to render any depleted nerve terminals visible Since the incubation procedure did not result in any appreciable alterations in the innervation patterns of the hearts, it can be assumed that the histochemical fluorescence method gave a true picture of the density and distribution of their adrenergic nervous systems The development of an apparently normal nervous system related to the newly formed blood vessels in the hearts from the swimming exercised (and hypertensive) rats was probably the sole basis for the increased density of fluorescent fibres observed in these hearts, since the number of nerve filaments per 100 capillaries was not altered

In cardiac hypertrophy secondary to aortic-al stenosis *Spann et al* (1965), *Krakoff et al* (1967) and *Meerson* (1969) reported a decrease in the norepinephrine content of the heart *Vogel et al* (1969) ascribed this to a decrease in the density of adrenergic nerves We found no evidence of this in our rats with aortic stenosis, but an unaltered innervation may well lead to a relative decrease in the norepinephrine content in such hearts since their muscle mass will be increased

In agreement with other authors (*Haebara et al* 1968) we noted an increased density of adrenergic nerve structures around the large myocardial arteries in hypertensive rats This nerve change may be a reaction to the increase in thickness of the muscle wall of these arteries (*Ljungqvist & Unge* 1972) A similar nerve reaction was, however observed in the swimming exercised rats in which the muscle walls of the myocardial arteries do not increase in thickness It is possible that the nerve reaction in these hearts is a feature of an adaptation to increased functional demand on the arteries

This study was supported by the *Swedish Medical Research Council* (Project No B72 12X-716 07A) the *Swedish National Association against Heart and Chest Diseases* and the *Research Funds of Karolinska Institutet*

REFERENCES

1 *Angelakos, E T, Fuxe, K & Torchiana, M L* Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts Acta physiol scand *59* 184–192, 1963

2 *Dahlstrom, A, Fuxe, K, Mya Tu, M & Zetterström, B E M* Observations on adrenergic innervation of dog heart Amer J Physiol *209* 689–692, 1965

3 *Dahlstrom, A & Zetterstrom, B E M* Noradrenaline stores in nerve terminals of the spleen Changes during haemorrhagic shock Science *147* 1583 1584, 1965

4 *Ehinger, B, Falck B & Sporrong, B* Adrenergic fibers to the heart and to peripheral vessels Bibl anat *8* 35–45, 1966

5 *Falck, B, Hillarp, H A, Thieme, G & Torp A* Fluorescence of catecholamines and related compounds condensed with formaldehyde J Histochem Cytochem *10* 348 354 1962

6 *Fuxe, K, Hokfelt, T, Jonsson, G & Ungerstedt, U* Fluorescence microscopy in neuroanatomy In Nauta, W & Ebbesson, J (Ed) Contemporary research methods in neuroanatomy Springer, 1970 pp 275 314

7 *Haebara, H, Ichijima K, Motoyoshi T & Okamoto, K* Fluorescence microscopical studies on noradrenaline in the peripheral blood vessels of spontaneously hypertensive rats Jap Circul J *32* 1391 1400, 1968

8 *Hamberger, B* Reserpine resistant uptake of catecholamines in isolated tissues of the rat A histochemical study Acta physiol scand suppl 295 1 56 1967

9 *Jacobowitz, D, Cooper, Th & Barner, H P* Histochemical and chemical studies of the localization of adrenergic and cholinergic nerves in normal and denervated cat hearts. Circ Res *20* 289–298 1967

10 *Krakoff L, de Champlain J & Axelrod J* Abnormal storage of norepinephrine in experimental hypertension in the rat Circ Res *21* 583–591 1967

11 *Krokhina E M* The adrenergic component of the effector heart innervation Histochemical study by the method of fluorescent microscopy Acta anat *74* 214–227, 1969

12 *Ljungqvist A & Unge G* The finer intramyocardial vasculature in various forms of experimental cardiac hypertrophy Acta path. microbiol scand Sect A *80* 329 340, 1972

13 *Ljungqvist A & Unge, G* The proliferative activity of the myocardial tissue in various

forms of experimental cardiac hypertrophy Acta path microbiol scand Sect A 81 233–240, 1973
14 *Ljungqvist, A* & *Ungerstedt, U* Symbathetic innervation of the juxtaglomerular cells of the kidney in rats with renal hypertension Acta path microbiol scand Sect. A 80 38–46, 1972
15
16 *Mandache, E*, *Unge, G*, *Appelgren, L E* & *Ljungqvist, A* The proliferative activity of the heart tissue in various forms of experimental cardiac hypertrophy studied by electron microscope autoradiography Virchows Arch Abt B Zellpath 12 112 122, 1973
17
18
ergic nervs. Histochemie 15 38–45, 1968
19 *Olson, L* & *Ungerstedt, U* A simple high capacity freeze drier for histochemical use Histochemie, 22 8–19, 1970
20 *Spann, J, Jr*, *Chidsey, Ch A*, *Pool, P E* & *Braunwald, E* Mechanism of norepinephrine depletion in experimental heart failure produced by aortic constriction in the guinea pig Circ Res 17 312–321, 1965
21 *Winckler, J* Über die adrenergen Herznerven bei Ratte und Meerschweinchen Entwicklung und Innervationsmunster Z Zellforsch 98 106–121, 1969
22 *Winckler, J* Katecholamingehalt adrenerger Herznerven der Albinoratte nach korperlicher
23
ing bovine heart Circ Res. 24 71–84, 1969
24 *Östman, I*, *Sjostrand, N O* & *Swedin, G* Cardiac noradrenaline turnover and urinary catecholamine excretion in trained and untrained rats during rest and exercise Acta physiol scand 86 299–308, 1972

Acta path microbiol scand Section A 81, 3, 376–378, 1973

BRIEF REPORTS

RAPID MARKED INCREASE IN PLASMA RENIN IN RATS TREATED WITH INHIBITORS OF THE RENIN SYSTEM

Effects of 1-Sar 8 Ala-Angiotensin II and of a Synthetic Converting Enzyme Inhibitor (Nonapeptide, SQ 20 881) on Normal and Adrenalectomized Rats

Jens Bing

It is known that continuous i v infusion of angiotensin II reduces renin release in both dogs (10, 3), man (4), sheep (2) and rat (7) It seemed therefore probable that blockade of the renin system with resulting decreased angiotensin formation would increase renin release Such an effect was recently shown by *Miller et al* (1972), who found that *renin activity* was more markedly elevated following renal artery constriction in dogs treated with a potent inhibitor of the converting enzyme than in untreated dogs

The present paper aims at studying the effect in normal and adrenalectomized rats of 1) a specific competitive inhibitor of angiotensin II (8) and of 2) an inhibitor of the angiotensin converting enzyme (for literature, see 6), both renin concentration, renin substrate and renin activity being determined.

Material and Methods

Female S P F Wistar rats (B W about 200 g) either normal or adrenalectomized and substituted with 1 per cent sodium chloride solution as drinking fluid and 0 5 mg desoxicortone acetate given s c in oil the first 4 days of the week, the dose being trebled the 5th day, while no injections were given the following 2 days With this substitution the renin values were somewhat elevated the first day after the DOCA free weekend, but on the follow-days uninfluenced by the adrenalectomy, which was performed on penicillin pretreated ether anaesthetized animals The infusions were performed on conscious animals at least 1½ hours after a short ether anaesthesia, during which a catheter was inserted into the jugular vein Blood was drawn from a carotic artery about 5 minutes after i p injection of 100 mg/kg amobarbital. The blood was cooled to 4° C adding 50 µl 6 per cent sodium citrate solution pr ml Plasma renin concentration and activity and renin substrate were determined by a slight modification of a radio immunoassay for angiotensin (*Poulsen* 1969), omitting converting enzyme and adding EDTA The material consists of 4 groups of rats, which were either infused with 0 1 ml per hour of 1) 0 9 per cent NaCl (controls), 2) 50 ng angiotensin II (hypertensin, Ciba) per min, 3) 1 2 µg 1 Sar 8 Ala Angiotensin II (Norwich Pharm Comp, New York) per min or received 4) a single injection of 0 4 mg of an angiotensin converting enzyme inhibitor a synthetic bradykinine potentiating nonapeptide, designated SQ 20,881 (Squibb Corp, Princeton)

The renin system was assayed twice 1) 24 hours before the experiments (–24 in the Fig), the values being marked △ in normal (norm) and ○ in adrenalectomized DOCA and salt substituted (adren) rats, and 2) after 5 min to 6 hours of infusion (▲ norm and ● adren)

Results

In the controls which were infused with 0 9 per cent NaCl (Fig 1 A) there is a tendency to a fall in renin concentration and activity in the norm but not in the adren rats while renin substrate was uninfluenced in both In the *angiotensin II infused rats* there was a marked fall in concentration as

strate as Fig 1A had to have 4 and 5 times higher ordinates for renin concentration and activity respectively It shows that *infusion of the competitive angiotensin II inhibitor* 1 Sar 8 Ala Ang II, results in very rapid and marked increases in both renin concentration and activity in *nearly* all adren rats where there were from 4 to 14 times increased values which could be found as *early as* 5 minutes after the start of the infusion *Contrary*

Received 5 iv 73 Accepted 9 iv 73

University Institute for Experimental Medicine
Nørre Alle 71, 2100 Copenhagen Ø, Denmark

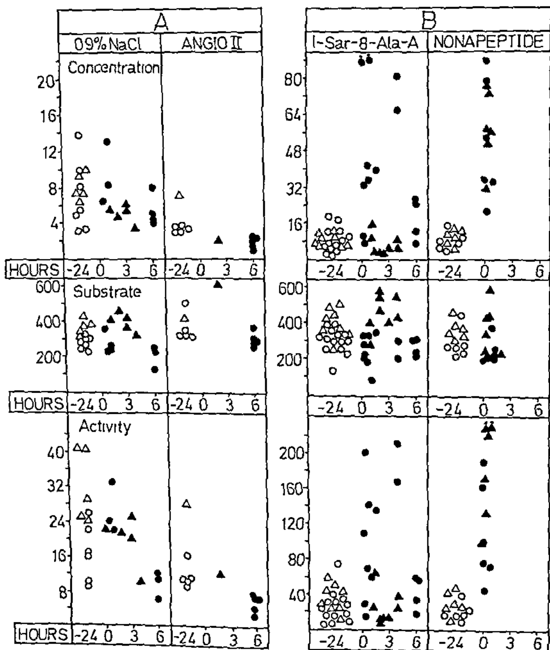


Fig 1 shows the *plasma renin concentration* (in hours $^{-1} \times 10^{?}$), *renin substrate* (in ng angiotensin II per ml) and *renin activity* (in ng $\times$ ml^{-1} $\times$ hours^{-1}) in rats infused with 0.1 ml per hour of solutions of either 0.9 per cent NaCl, angiotensin II or the competitive angiotensin II inhibitor 1 Sar 8-Ala Ang II, or injected with a solution of the converting enzyme inhibiting nonapeptide (SQ 20,881). The symbols are △ for normal and ○ for adrenalectomized DOCA and salt substituted rats, when they express the values 24 hours before *start of the treatment* (–24), and ▲ and ● for values measured from 5 minutes to 6 hours after the start. The differences in the ordinates in Fig 1 A and 1 B should be noted.

to this the inhibitor neither changed concentration nor activity in norm rats The substrate was unchanged in the adren and only slightly increased in the normal rats The effect of the *converting enzyme inhibiting nonapeptide* differed from that of the angiotensin II inhibitor, giving both in norm and adren rats a rapid and marked (2 to 18 times) increase in renin concentration as well as in renin activity It did not produce any change in renin substrate

Discussion and Summary

In accordance with previous studies (10, 3, 4, 2, 7) the present paper shows that there is a negative feedback mechanism between plasma angiotensin and renin release, both renin concentration and activity falling during i v infusion of *angiotensin II* (Fig 1 A) The opposite direction of the same negative feedback mechanism is shown, when the formation of angiotensin II is blocked by injection of *the converting enzyme inhibiting synthetic nonapeptide (SQ 20,881)*, which is followed by a rapid marked increase in renin concentration as well as in renin activity in both normal and adrenalectomized DOCA and salt substituted rats (Fig 1 B) Less predictable is the effect on renin release of the *competitive angiotensin II inhibitor 1-Sar 8 Ala Ang II*, which does not act by decreasing plasma angiotensin (which may perhaps even be somewhat increased), but by blocking the receptor sites (8) Although this is so, the angiotensin II inhibitor gives about the same rapid and marked increase in plasma renin concentration and activity as the converting enzyme inhibitor, this however only being the result in the adrenalectomized, but not in the normal rats (Fig 1 B) A somewhat similar result was quite recently obtained by *Johnson & Davis* (1973), who with the same angiotensin II blocker found increased renin activity in dogs with thoracic inferior vena cava constriction, but not in normal dogs

While angiotensin II infusion results in an elevation of *renin substrate* in normal rats (7), the substrate is unchanged in angiotensin infused adrenalectomized rats (Fig 1 A) It is further seen (Fig 1 B), that the substrate is not depressed when renin is markedly elevated after treatment with inhibitor The mechanism of the angiotensin induced increase in substrate found in normal rats can be similar to that of different kinds of "unspecific" adrenal dependent increases both in renin substrate and in acute phase proteins (1)

Supported by a grant from *King Christian X Foundation* and kind gifts of inhibitors from Dr *A Castellion,* The Norwich Pharm Co, and Dr *F Giarusso,* The Squibb Institute

References 1 *Bing, J* This journ *80* 646–650 1972 —2 *Blair West, J R, Coghlan, J P, Denton D A, Funder, J W, Scoggins, B A & Wright, R D* Am J Physiol *220* 1309–1315, 1971 —3 *Bunag, R D, Page, I H & McCubbin, J W* Cardiovasc Res *1* 67 73, 1967 —4 *Genest, J Champlain, J de, Boucher, R, Veyrat, R & Koiw E* Path Biol *13* 1024–1037, 1965 —5 *Johnson, J A & Davis, J O* Science *179* 906 907, 1973 —6 *Miller Jr E D, Samuels, A I Haber, E & Barger, A C* Science *177* 1108 1109, 1972 —7 *Nasjletti, A & Masson, G M C* Proc Soc Exp Biol Med *142* 307–310 1973 —8 *Pals, D T, Masucci, F D Denning, G S, Sipos F & Fessler, D C* Circ Res 29 664 681, 1971 —9 *Poulsen, K* Scand J Clin Lab Invest *24* 291–300 1969 —10 *Vander, A J & Geelhoed G W* Proc. Soc Exp Biol Med *120* 399–403, 1965

Acta path microbiol scand Section A 81, 3, 379-380, 1973

MYCOPLASMOSIS EXPERIMENTAL PYELONEPHRITIS IN RATS

A C Thomsen, S Rosendal and *O Frøkjær Thomsen*

Mycoplasmas in man occur frequently in the lower urinary tract A few reports suggest that they may be found in the upper urinary tract as well, but *their pathogenic role is not clear* (2, 4)

Rats have been widely used as an experimental model in investigations of bacterial infections in the upper urinary tract and the present work reports on analogous experiments with *Mycoplasma arthritidis*

Material and Methods

Rats White Spraque Dowley, SPF rats (male), weight 200 300 gr were used

Strain of Mycoplasma Strain 150 P 10 of *M arthritidis, the arthritogenic pathogenicity of which* had been increased through serial passages in rats (1), was used

Cultivation was made in 500 ml of Hayflick's medium without penicillin and thallium acetate The culture was centrifuged at 34,000 × g in a Sorvall refrigerated centrifuge (RC 2B) The sediment was resuspended in 50 ml of PBS pH 7 4 The number of colony forming units per ml (c.f u /ml) was determined to 2×10^9 The suspension was stored in volumes of 1 ml at 70° C

Ligation of ureter Rats were anaesthetized by intra peritoneal injection of 0 1 ml Nembutal® (50 mg/ml) supplemented with 0 1 ml (5 mg/ml) Stesolid® After shaving and cleaning of the epidermis with 70 per cent ethanol the abdomen was opened by a 2 cm incision parallel to and about 1 cm to the left of the lumbar column About 1 cm distal to the ureteropelvic junction a ligature (3 0 merseline) was placed around the ureter The open ends of the ligature were passed through the left flank and tied around a piece of latex drain The incision was sutured in layers After 20 hours the ligature was cut and removed

Inoculation Immediately after ligation of the ureter, 1 ml containing 2×10^9 c f u /ml *M arthritidis* (158 P10) was injected intracardially Controls were injected intracardially with 1 ml PBS

Post mortem examination The rats were euthanized with 0 2 ml Nembutal® (50 mg/ml) intraperitoneally after 5 days Both kidneys were removed aseptically For quantitative cultivation of mycoplasmas and bacteria, half a gram of kidney tissue was homogenized in 4 5 ml PBS Cultivation was performed on Hayflick's medium and blood agar

For histology and immunofluorescence examination, kidney tissue was frozen at −70° C in isopentan whereafter the tissue was sectioned (4μ) on a cryostat The sections were air dried for two hours and every fifth section was stained with haematoxylin eosin for histological examination The remaining sections were used for immunofluorescence examination by the indirect method One drop of *M arthritidis* (PG 6) rabbit anti serum diluted 1 10 was placed on every second section Neighbouring sections were treated with one drop of normal rabbit serum diluted 1 10 Following incubation for 30 min at room temperature, the sections were washed 2 × 10 min in PBS pH 7 4 and then treated with one drop each of FITC conjugated anti rabbit immunoglobulin diluted 1 50 Finally the sections were incubated for 30 min at room temperature washed 2 × 10 min in PBS pH 7 4 and mounted in glycerol PBS (9 to 1) Fluorescence was studied in a Reichert Zetopan fluorescence microscope

Results

A total of eight rats were inoculated with *M arthritidis* The unobstructed kidneys were invariably sterile and without macroscopically or histologically demonstrable lesions.

From the obstructed kidneys *M arthritidis* could be cultivated in a number of $10^4 - 4 \times 10^6$ c f u./gr

Received 9 iii 73 Accepted 9 iii 73

FAO/WHO International Reference Centre for Animal Mycoplasmas The Institute of Medical Microbiology University of Aarhus, and the University Institute of Pathology, Kommunehospitalet DK 8000 Aarhus C Denmark

Request for reprints should be addressed to *A C Thomsen*, The Institute of Medical Microbiology University of Aarhus, DK 8000 Aarhus C Denmark



Fig 1 Section of the left kidney from rat inoculated with *M arthritidis* Typical absces in the papilla Haematoxylin eosin × 160

Fig 2 Specific fluorescence in the absces Stain for *M. arthritidis* (PG 6) × 400

tissue Bacteria could not be cultivated Macroscopically the kidneys were moderately enlarged (hydronephrosis), but without abscesses Histological examination revealed focal inflammatory lesions in the obstructed kidneys, predominating in the papilla where fully developed abscesses, sometimes with ulceration and necrosis, were seen In a few cases, minute abscesses were seen in the cortex The abscesses were surrounded by enlarged tubules stuffed with neutrophilic granulocytes and hyaline casts The tissue surrounding the abscesses showed varying degrees of fibrosis (Fig 1)

The kidneys from 4 control rats were sterile Except for hydronephrosis, neither macroscopic nor microscopic lesions were present

Immunofluorescence microscopy was carried out on the obstructed kidneys from two rats inoculated with *M arthritidis* In both cases, strong fluorescence was demonstrable in sections treated with *M arthritidis* (PG 6) antiserum (Fig 2)

The fluorescence was found in an area of the papilla showing heavy infiltration of neutrophilic granulocytes if examined in sections stained with haematoxylin eosin No fluorescence was seen in control sections treated with normal serum

Discussion

The well known capability of *M arthritidis* to produce suppurative lesions in rats motivated the choice of this organism for the present study (3)

Using *M arthritidis,* the experimental production of acute pyelonephritic lesions in rat kidneys with complete obstruction of the ureter, as opposed to the absence of any lesions in non obstructed kidneys under identical conditions suggests that obstruction of the urinary tract is essential for the colonization and pathogenic effect of mycoplasmas in the kidney It may be speculated that mycoplasmas may play an aetiological role, in themselves or in association with bacteria, for the development of pyelonephritis in connection with naturally occurring obstructive conditions of the urinary tract in animals and man

The experimental model described in this study may contribute, it is believed to elucidating further the potential pathogenicity of certain human mycoplasmas and to studying the possible role of mixed infections with mycoplasmas and bacteria in pyelonephritis

References 1 *Golightly Rowland, L , Cole, B C , Ward, J R & Wiley, B B* Infect and Immun *1* 538 545 1970—2 *Purcell, R H* Ann N Y Acad Sci *170* 786–793 1970—3 *Ward, J R & Cole, B C* In *Sharp J T* (Ed) The Role of Mycoplasmas and L Forms of Bacteria in Disease Charles C Thomas, Springfield, Illinois, U S A 1970 p 212–239—4 *Weileb, W , Farber, I , Thieler, H & Blumöhr, T* Zbl Bact , I Abt. Orig *208* 427–430, 1968

Acta path microbiol scand Section A. 81 381-389, 1973

MULTIPLE EPIPHYSEAL DYSPLASIA, WITH SPECIAL REFERENCE TO HISTOLOGICAL FINDINGS

P G RASMUSSEN and I REIMANN

Small Animal Clinic Royal Veterinary and Agricultural University, and Orthopaedic Hospital Copenhagen, Denmark

Radiological and clinical comparisons of multiple epiphyseal dysplasia were made in four children and five puppies On the basis of these, it is assumed that the disease in children and puppies is closely related Two six week-old beagle puppies with epiphyseal dysplasia were examined histologically It was found that bone changes developed primarily in the hyaline cartilage Two forms of pathological tissue were demonstrated in the cartilage One was an acellular structureless mass which underwent secondary calcification The other form was located outside the first and consisted of abnormal intercellular calcified hyaline cartilage Bones with pronounced stippled radiographical appearance (punctate epiphyseal dysplasia) showed massive calcifications of clear cut demarcated acellular masses Bone with pronounced mottled radiographical appearance (multiple epiphyseal dysplasia) showed zones of abnormal intercellular calcified hyaline cartilage However, both forms of pathological tissue were found in bones with radiographical stippled calcification and in those with radiographical mottled calcification

Multiple epiphyseal dysplasia, "stippled epiphyses", or chondrodystrophia calcificans congenita, is a rare though well known disease in children The condition has been reviewed from clinical and radiological aspects (*Fairbank 1951*, *Rubin 1964*, *Mosekilde 1968*), but reports of the histological changes are few

Reports of the disease are also rare in the veterinary literature, where only few accounts of the condition in puppies have been published (Cotchin & Dyce 1956, Lodge 1966 Rasmussen 1971) In children, the disease is congenital and in severe cases is characterized clinically by deformity of the extremities (often with multiple contracture) and unilateral or bilateral cataract. Stippling can be seen on the radiograph already at birth, and this may be present universally in the cartilage bones Stippling can often be found in the carpal and tarsal bones, and in some cases the changes are localized there exclusively (*Svoboda 1950 Fairbank 1951*)

In puppies, similar radiological findings have been demonstrated in cases where there were clinical signs of difficulty in walking Accompanying cataract has not been reported

The aim of the present study was to compare the radiological changes found in children and puppies and to describe the histological changes occurring in multiple epiphyseal dysplasia in puppies

Received 28 x 72 Accepted 9 1 73

Requests for reprints should be addressed to *P G Rasmussen* Royal Veterinary & Agricultural University, Bulowsvej 13 DK 1870 Copenhagen V, Denmark.

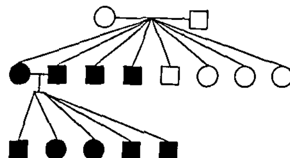


Fig I Beagle population in three generations the first subjected to clinical examination, the second and third to clinical and X ray examination □ normal male, ○ normal female, ■ male with epiphyseal dysplasia, ● female with epiphyseal dysplasia

MATERIAL AND METHODS

Four children (three girls and one boy) and five beagle puppies (two females, three males) were observed clinically and radiologically from birth Radiographs were taken at intervals of three to six months in the case of the children, and as regards the puppies once a week at the beginning and later once a month No definite familial disposition to the disease could be demonstrated in the children. The five beagle puppies were all of the same parents, both of which had the disease The parents were from a litter of eight, four of which had the disease (Fig I)

Histological preparations were made from two of the beagle puppies Labelling was made with Calcein (20 mg per kg) and Alizarin (30 mg per kg) three days and one day before the puppies were killed at the age of 42 and 54 days The whole skeletal system was fixed in ethanol and preparations were made from the left elbow and left tarsal bones of both animals The specimens were embedded in methyl metacrylate without previous decalcification and then cut by a Jung microtome into sections with a thickness of 5 μ The sections were stained with haematoxylin eosin, toluidine blue and von Kossa for light microscopy Unstained sections were used for fluorescence microscopy

RESULTS

The stippled calcification could be demonstrated radiologically in the four children at

TABLE 1 *Localization of Abnormal Calcifications in the Epiphyses and Cuboid Bones of Children and Puppies*

	Child				*Beagle puppy*				
	1	2	3	4	1	2	3	4	5
Femur	×	×			×	×	×	×	×
Tibia	×	×							
Fibula	×	×							
Calcaneus	×	×	×	×	×				×
Talus	×	×	×		×				×
Ossa tarsalea	×	×			×	×			×
Metatarsus	×	×							
Phalangis									
Scapula	×	×						×	
Humerus	×	×			×	×	×	×	×
Radius	×	×							
Ulna	×	×							×
Ossa carpalea	×	×			×	×			×
Metacarpus	×	×							×
Columna coccygealis									
Columna lumbalis	×	×			×			×	
Columna thoracalis	×	×							
Columna cervicalis	×	×							
Pelvis	×	×							
Costae	×	×							
Sternum	×	×							
Cranium									
Larynx	×	×							



Fig II Punctate calcification in the tarsal bones of (1) a one month old girl and (2) a six week-old beagle puppy

birth, and in the beagle puppies the first radiological findings appeared at the age of three weeks

The development of the calcifications and the subsequent incorporation into the normal ossification of the bones were found, on the basis of radiographs, to take three to five years in the children, while in the puppies the changes could be observed from the third week to the fifth month

As will be seen from Table 1, the radio logical anomalies in two of the children were found in all the cartilage bones and in the laryngeal cartilage while the other two children showed radiological changes in the tarsus only As regards the beagle puppies, the abnormalities were found particularly in the epiphyses of the femur and humerus and in the tarsal and carpal bones The striking similarity between the abnormal calcification in the children and the puppies can be seen from Fig II, which shows the processes in the tarsal bones

Evaluation and Description of the Histological Preparations

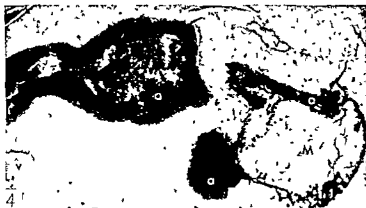
The specimens for determination of the histological changes were taken both from the tarsus and elbow joints, so that it was possible to assess the situation in a cuboid bone and in an epiphysis of a tubular bone

As demonstrated radiologically, histological examination of the tarsus revealed an ossification centre in the middle of the bone (Fig III) This ossification centre showed a normal cartilaginous growth zone with the cartilage cells arranged into columns, and normal primary calcification of the intercellular substance In the middle of the ossification centre there were normal bone trabeculas In the surrounding cartilage there were foci of pathological tissue measuring up to 1 mm These areas contained abnormal calcifications varying from a few disseminated deposits (Fig III) to massive calcification of the whole area (Fig IV)

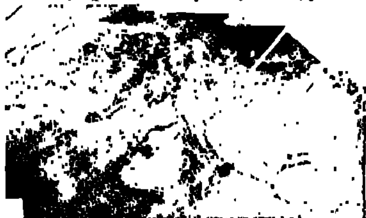
In the foci with little calcification, two forms of abnormal tissue could be demonstrated One consisted of an acellular structureless mass which stained red with toluidine blue Scattered around in this structureless mass were calcium deposits which were most numerous at the periphery Adjacent to the structureless mass was either normal or pathological hyaline cartilage However, the chondrocytes in the normal cartilage were sometimes flattened and the perifocal tissue was often conspicuously vascularized The other form of pathological tissue consisted of abnormal cellular hyaline cartilage with dense areas of calcium deposits in the intercellular substance This tissue lay always in a zone outside the acellular structureless mass In the preparations from the tarsus, this abnormally calcified hyaline cartilage covered only a relatively thin zone and was seen in only a few of the foci

The foci with much calcification (Fig IV) contained the same two forms of pathological tissue, one being acellular and the other cellular In the acellular form, there were islands or streaks of structureless acellular mass in between the calcium deposits The cellular form contained cavities with degenerated chondrocytes

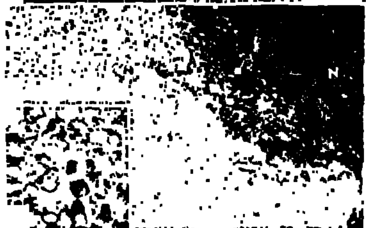
The foci with both forms of pathological tissue always showed a sharp demarcation line between the inner acellular mass with the varying amounts of calcification and the outer zone of abnormal cellular cartilaginous tissue. Furthermore, there was a clear distinction between the acellular mass and the surrounding hyaline cartilage


M
4






2


N


T
1


M
N

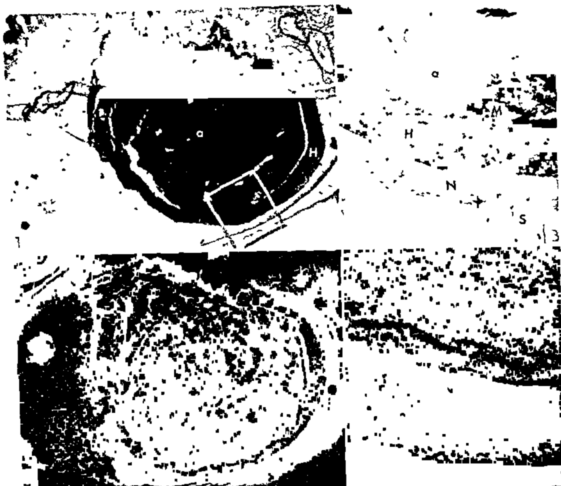


Fig IV Solid punctate calcification lying distally in the cartilage of the tarsal bones, shown in Fig III, 2, 3 Inside is a large area of solid calcifications (a) of acellular masses (M) and at the periphery a narrow zone of calcified hyaline cartilage (H) There is a clear-cut division between normal articular cartilage (N) and calcified hyaline cartilage (H) Articular space (S) Myxoid degeneration (d) shown in Fig VI A corner of the ossification centre of the tibial tarsal bone (T) Vessels (v) Fall out of a punctate calcification (f) (1) von Kossa, (2) toluidine blue, magnification 50 ×, (3) and (4) magnification 200 ×

Fig III Tarsal bones from a six week-old beagle puppy

1, 2 & *3* Normal ossification centres in the talus (T) and calcaneus (C) Abnormal calcifications (a) shown in Fig IV (1) radiograph, (2) toluidine blue, (3) von Kossa, undecalcified, magnification 15 ×

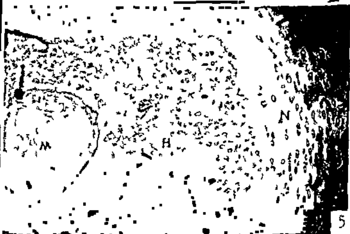
4 & *5* Pathological tissue consisting of acellular pathological mass (M) with abnormal calcifications (a) The calcifications are most intense at the periphery of the foci Some large vessels (v) Area with preparation fall-out (f) (4) von Kossa, (5) toluidine blue, magnification 50 ×

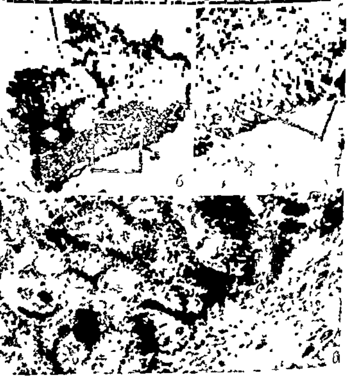
6 Clear-cut division between the pathological tissue and normal cartilage (N) The calcium deposits (a) are very dense at the periphery of the acellular pathological masses (M) Toluidine blue, magnification 200 ×

7 Calcium deposits in the acellular pathological masses. Toluidine blue, magnification 800 ×

8 Clear-cut division between acellular pathological masses (M) and normal cartilage (N) No column formation of the slightly flattened chondrocytes. Some dense calcium deposits (a) at the edge of the acellular masses. Toluidine blue, magnification 800 ×





The chondrocytes in the normal hyaline cartilage adjacent to the foci were irregularly arranged and did not show the column formation seen in the normal ossification centres

There was no fluorescence in the calcified tissue in the pathological cartilaginous foci lying isolated in the normal hyaline cartilage, nor any other histological signs of an ossification process However, in the abnormal calcifications located at the edge of the ossification centres, there were a few fluorescent streaks or spots Active osteoclasts and osteoblasts could be seen and there was increased vascularization Where the ossification centre incorporated the abnormal calcified cartilaginous focus, the bone beams seemed to be deformed

The radiological anomalies of the epiphysis of the humerus were different from those in the tarsal bones at the time of examination,

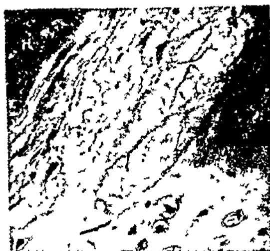


Fig 11 Magnification of Fig IV, 2, d, myxoid degeneration of hyaline cartilage Abnormal calcified hyaline cartilage (H) Toluidine blue magnification 800 ×

since the abnormal calcifications in the humerus showed more diffuse mottled shadows The outlines of the ossification centres of the condyles were irregular and were blurred by the abnormal calcification (Fig V) Histological examination revealed a normal growth plate with chondrocytes arranged in columns and primary calcifications in the intercellular substance The pathological cartilaginous processes were in the epiphysis outside the growth plate As in the tarsus there were two forms of cartilaginous anomalies, viz an acellular and a cellular form (Fig V) Inside was an acellular structureless mass which stained red with toluidine blue It was not possible by haematoxylin eosin staining to demonstrate any difference in the staining affinity of the abnormal homogeneous mass and the normal cartilaginous intercellular substance There were diffuse calcium deposits in the acellular mass which as in the case of the tarsus were most numerous at the periphery

Around the acellular mass in the epiphysis of the humerus was a relatively broad zone of pathological hyaline cartilage in which could be seen marked granular calcium deposits in the intercellular substance The chondrocytes lay irregularly without column formation

Fig V Elbow joint of a six week-old beagle puppy

1 Calcifications in the humeral epiphysis (E) with a very irregular and mottled feature radiographically Magnification 15 ×

2 In the cartilage of the humeral epiphysis (E) is a large area of acellular pathological mass (M) which penetrates the articular cartilage anteriorly Area with preparation fall out (f) Toluidine blue undecalcified magnification 15 ×

3 Two tangentially sectioned foci of abnormal calcification (a) in the cartilaginous humeral epiphysis (E) Ulnar bone with normal trabecular structure (U) Von Kossa undecalcified magnification 15 ×

4 Very large area of acellular pathological tissue with a clear-cut division between the surrounding abnormal calcified hyaline cartilage (H) Clear-cut demarcation and intense reddish stained area of the acellular pathological tissue (r) Paler reddish stained areas (p) Toluidine blue magnification 50 ×

5 Around the acellular masses (M) is a wide zone of abnormal calcified hyaline cartilage (H) with multiple fine granular deposits in the intercellular substance The abnormal calcified hyaline cartilage is clearly divided from the normal hyaline cartilage (N) Toluidine blue magnification 200 ×

6 7 & 8 Demonstration of the granular calcium deposits in the intercellular substance Note the sharp demarcation between cellular pathological tissue and normal hyaline cartilage Von Kossa magnification 50 200 and 800 ×

The calcium deposits in the intercellular substance were also located irregularly and had a granular appearance, in contrast to the compact appearance of the primary calcification in a normal ossification centre Here too there was a sharp line of demarcation between the acellular mass and the pathological hyaline cartilage and between the pathological hyaline cartilage and the normal cartilage

Fluorescent components could not be demonstrated in the two forms of pathological tissue in the epiphysis of the humerus It could be seen in sections from the humerus that the pathological tissue penetrated the joint cartilage in places Only in one of the

DISCUSSION

Comparison of the results of the radiological examinations in the present study with those reported in the literature (*Fairbank* 1951, *Mosekilde* 1968, *Silverman* 1961, *Rubin* 1964 *Brandrup* 1970) showed that there is a close similarity between the bone anomalies in children and in puppies

The disease is demonstrable radiologically at birth in children and in puppies at the age of about three weeks This is in agreement with the juvenile development of the child and the puppy *Frank* & *Denny* (1954) described a case in which the disease was diagnosed by radiograph antenatally on a 7-month-old human foetus Analogous with the development and disappearance of the stippled anomalies, these can be demonstrated in children within the first three to five years and in puppies within the first five months

The disease manifests itself with varying phenotypic expressions in children and puppies. In some cases all or the majority of the cartilage bones are affected, whereas in others the disease attacks single bones only On this account it may be assumed that multiple epiphyseal dysplasia in children and in puppies is closely related

Hereditary disposition has been reported in humans (*Raap* 1943, *Bergström et al* 1971) In the present study, no familial tendency could be demonstrated with any certainty As regards the beagle puppies in the present study, the disease was hereditary and probably autosomally recessive (Fig 1)

It is obvious from comparison of the radiological and histological findings that it is the calcifications in the pathological foci in the cartilage which cause the stippled or mottled appearance on the radiographs

The pathological cartilaginous foci in the epiphyses were found in the part of the cartilage that forms bone from the ossification centre of the epiphysis This corresponds to the findings in the cuboid bones where the foci lie in the cartilage around the ossification centre No cartilaginous growth zone was demonstrated around the foci and thus there can be no question of subsidiary ossification centres as suggested by the radiological examination (*Conradi* 1914)

Evaluation of the radiographs shows both typical stippling and cases with a more mottled appearance When compared with the histological findings, the stippling corresponds to the extensively calcified acellular foci in the cartilage, and the mottling to the broad zones of calcification in the hyaline cartilage at the periphery of the acellular mass Based on radiological examinations, *Silverman* (1961) described two forms of the disease, viz punctate epiphyseal dysplasia and multiple epiphyseal dysplasia His conclusion was that these are the same disease but that there is a difference in degree This corresponds with the findings in the present study Multiple epiphyseal dysplasia should therefore not be classified into two separate groups, as suggested by *Rubin* (1964), where one form shows radiological punctate calcifications and is congenital, and the tardive form gives mottled calcifications and occurs in older children

The histological examinations indicate that the bone changes begin primarily in the hyaline cartilage Based on biopsies from children, *Tisdall* & *Erb* (1924), *Bateman* (1939) and *Frank* & *Denny* (1954) reported anom

alies corresponding to myxoid degeneration Such a condition was found in the present study only in one preparation from the tarsus *Cohen Solal et al* (1968) demonstrated in histochemical studies that there was a reduction in the number of acid mucopolysaccharides corresponding to the cartilaginous processes The red staining of the foci with toluidine blue also suggests a biochemical change

The further development of the epiphysis and the ossification of the cuboid bones result in the incorporation of the abnormal calcifications into the ossification centre From there there is an ingrowth of vessels and both osteoblast and osteoclast activity occur The calcified foci then change into deformed bone trabeculas which gradually become remodelled into normal At that late stage there are no specific radiological changes However, it is obvious that the bone, and particularly its articular surfaces during growth, may become more and more deformed because of the extensive degeneration in the cartilage which hinders normal growth and development

REFERENCES

Bateman, D Two cases and specimens from a third case of epiphyseal dysplasia puncticularis Brit J Child Dis. *36* 219–220, 1939

Bergstrom, K, Gustavson, K H, Jorulf, H, Melander, S & Thor, L Chondrodystrofia calcificans congenita i ny genetisk belysning Lakartidn *68* 3023–3026, 1971

Brandrup, Fl O Chondrodystrophia calcificans congenita Ugeskr Læg *20* 937–939, 1970

Conradi, E Vorzeitiges Auftreten von Knochen und eigenartigen Verkalkungskernen bei Chondrodystrophia fotalis hypoplastica Histologische und Rontgenundersuchungen Jahrb Kinderh *80* 86–97, 1914

Cohen Solal, J, Faure, Cl, Sarut, S, Loubry, P, Moreno, J L, Lejeune, Cl & Mande, R Un cas de forme majeure de maladie des épiphyses ponctuees avec etude histologique Ann Pediat *15* 697–705, 1968

Cotchin, E & Dyce, K M A case of epiphyseal dysplasia in a dog Vetr Rec *68* 427–428, 1956

Fairbank, T A T An Atlas of General Affections of the Skeleton E & S Livingstone, Edinburgh and London, 1951, p 91–110

Frank, W W & Denny, M B Dysplasia epiphysia lis punctata J Bone & Joint Surg *36* B 118–122, 1954

Lodge, D Two cases of epiphyseal dysplasia. Vetr Rec *79* 136 138, 1966

Moseklide E. Chondroangiopathia Calcarea seu Punctata Handbuch der med Radiologie Springer Verlag, Berlin, Band V/3, 49–67, 1968

Raap, G Chondrodystrophia calcificans congenita Am J Roentgenol *49* 77–82, 1943

Rasmussen, P G Multiple epiphyseal dysplasia in a litter of Beagle puppies J Small Anim Pract *12* 91 96, 1971

Rubin, Philip Dynamic Classification of Bone Dysplasias Chicago, Year Book Medical Publishers, Inc 1964, p 120–159

Silverman, F N Dysplasies épiphysaires Entite protéiforme Ann radiol *4* 833–867, 1961

Swoboda, W Beitrag zur Chondrodystrophia calcificans congenita. Ann Paediat *175* 322–342, 1950

Tisdall, F E & Erb, I H Reports of two cases with unusual calcareous deposits Am J Dis Child *27* 28–38, 1924



Fig 3 Selected Area Diffraction Pattern taken from the Chrysotile Fibres in Fig 2



Fig 4 Amphibole Fibres Detected in Case No. 186 × 2 000

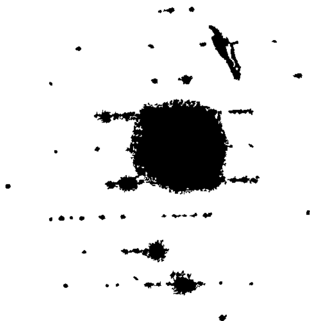


Fig 5 Selected area Electron Diffraction Pattern taken from single fibres in Fig 4

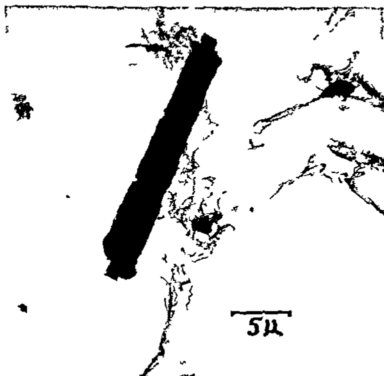


Fig 6 Amphibole Asbestos Body Case No 340 × 2 000

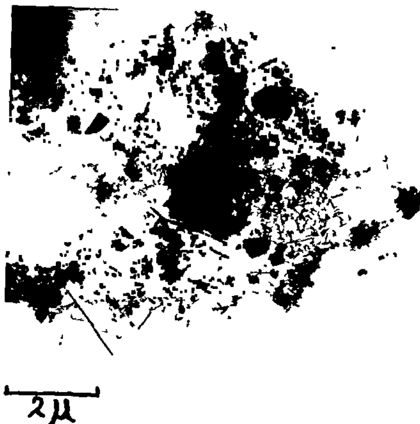


Fig 7 Typical Fine Chrysotile Fibrils found in Case No 1004 × 11 000

were found to contain asbestos 5 of the 9 cases contained Amphibole fibre alone, 3 contained *Chrysotile*, 1 a mixture of both types In total 28 of the 65 mesothelioma and control cases examined in the electron microscope were found to be positive for asbestos bodies by *Hagerstrand et al* Twelve of these contained Amphibole alone 8 contained Chrysotile, 5 a mixture of both types The 28 asbestos body positive cases were therefore found to be 89 per cent positive for asbestos 61 per cent containing Amphibole, 43 per cent containing Chrysotile

The detection of fibres in the ashed residue of thin sections is comparatively simple because of their distinct morphology The identification of the various types of fibres found is, however, more difficult with the exception of Chrysotile fibres which give distinct diffraction patterns The Amphibole varieties of asbestos fibre also give distinct diffraction patterns but it is not possible to distinguish readily between the varieties, i e Amosite and Crocidolite Figs 2 5 are examples of the diffraction patterns obtained from fibres of the two types found in some of the cases examined It can be seen that there is a distinct morphological difference between Chrysotile and Amphibole fibres as well as a marked difference in the character of the electron diffraction pattern

All the Amphibole fibres found occurred as single discrete fibres sometimes with a coating when enclosed in a body of the type shown in Fig 6 The majority of the Chrysotile fibres found occurred as small single fibres or bundles of fibres but large aggregates of several hundred fine fibres were also observed The appearance of Chrysotile fibres, fibre bundles and aggregates are illustrated by Figs 7 8, 2 The Amphibole fibres detected varied in size from 1 30 microns in length, while the diameters of the fibres did not exceed 0 6 microns The majority of the fibres found had diameters below 0 25 microns These diameters are consistent with the range of

Fig 8 Chrysotile Fibre Bundles Found in Case *No* 232 × 8 500



fibre diameters formed by the commercial *Amphibole minerals*, Amosite and Crocidolite In 3 of the cases very fine fibres were detected, which from their diffraction patterns, could not be categorized immediately as Chrysotile or Amphibole fibres It is suspected that these fine fibres were originally Chrysotile, which had been altered mainly by the leaching of the magnesium from the fibre

Although the ashing procedure adopted has a tendency to destroy most of the anatomical detail of the thin sections, the position of fibres relative to the tissue ash still reveals the clost association that some of the fibres had with the original tissue

None of the 65 cases examined can be considered to have had any significant exposure to asbestos dust

The cases observed with the largest quantities of asbestos fibre were all contained in the mesothelioma group

Only 4 of the 65 cases examined were found to have asbestos bodies, i e formations containing a detectable fibre core. *Hägerstrand et al* found 28 of the 65 to contain asbestos bodies This difference is not surprising, however, as the detection of the 28 positives was obtained from the examination of two 30 micron thick sections of each case under the light microscope The examination of each case with the electron microscope was made upon only five 3 mm diameter electron microscope grids prepared from a 6 micron thick section of each case The volume of material examined for fibres was therefore almost one hundred times smaller than that examined by *Hägerstrand et al* for asbestos bodies

CONCLUSIONS

Single Asbestos fibres are readily detected in lung tissue with an electron microscope, but with the exception of Chrysotile asbestos which is easily identified by its electron dif

6*

fraction pattern, it is not possible to distinguish between the Amphibole asbestos minerals No other mineral particles were detected which could possibly be confused with asbestos although in one or two cases fine fibrils were found resembling Chrysotile which were not positively identified The large majority of the Amphibole fibres found in the 65 cases examined possessed a morphology characteristic of Amosite and Crocidolite fibres Taking the 65 cases as whole, Chrysotile was found to be the most common asbestos mineral detected The mesothelioma cases were found to contain more fibres than the controls and also there a larger number of cases positive for asbestos than the controls The most common asbestos fibres found in those cases which were positive for asbestos bodies by light microscopy were the Amphibole types The results show that asbestos fibre in very small quantities is a common mineral in the lungs of the general population of most industrialised societies

The Author would like to thank Dr *I Hagerstrand* and Dr *L Meurman* for kindly allowing him to examine their specimens and also to thank Dr *J C Gilson* and Dr *J C Wagner* for their help in preparing this paper The investigation was financed by *British Medical Research Council* and also by the *International Agency for Research on Cancer*

REFERENCES

1 *Hagerstrand, I, Meurman, L & Odlund B* Asbestos Bodies in the Lungs and Mesothelioma Acta path microbiol scand 72 177 191, 1968

2 *Pooley, F D* Electron Microscope Characteristics of Inhaled Chrysotile Fibres Brit J Industr Med *29* 146–153, 1972

Acta path microbiol scand Section A 81, 401–410, 1973

EXPERIMENTAL MURINE LEPROSY

I Clinical and Histological Evidence for Varying Susceptibility of Mice to Infection with Mycobacterium lepraemurium

Otto Closs and Olav A Haugen

Institute for Experimental Medical Research, and Department of Pathology, Ullevål Sykehus, University of Oslo, Oslo, Norway

To propagate bacilli, a suspension of *Mycobacterium lepraemurium* (MLM) was injected intravenously into 40 outbred albino mice Pronounced differences were observed in the rate of progression of the infection, and 8 mice representing the clinically observed extremes were histologically examined Poor clinical condition was found to correlate with the presence of many granulomas and few or no surrounding lymphocytes, indicating weak or absent cell mediated immunity (CMI) Good clinical condition correlated with smaller and fewer granulomas with less bacilli and a pronounced infiltration of small lymphocytes. These observations strongly suggest that outbred mice differ in their susceptibility to MLM-infection The basis for this heterogeneity, which bears some resemblance to the spectrum in human leprosy, is assumed to be the varying capacity of individual mice to mount a cell mediated immune response against the mycobacterium

There is a wide spectrum of clinical disease in human leprosy, ranging from lepromatous leprosy (LL) at one end to tuberculoid leprosy (TT) at the other (*Ridley & Jopling* 1966) Being entirely an intracellular process, the proliferation of *Mycobacterium leprae (M leprae)* is expected to be inhibited by cell mediated immune reactions rather than by humoral antibody (*Mackaness* 1971a) This concept accords well with the close correlation between intensity of cell mediated immune reactions against *M leprae* and the clinical course in human leprosy, towards the lepromatous end of the spectrum there are increasing amounts of bacilli in the lesions and a concomitant decrease in cell mediated immunity (CMI) against the mycobacterium (*Turk & Bryceson* 1971, *Godal et al* 1971)

Received 15 i 73 Accepted 15 ii 73

Requests for reprints should be addressed to Institute for Experimental Medical Research, Ullevål Sykehus, Oslo 1 Norway

Circulating antibodies against mycobacterial antigens are usually absent in tuberculoid patients, but are regularly found in high titres in lepromatous patients and do not seem to have a protective effect (*Rees et al* 1965) The reason why individuals react so differently to infection with *M leprae* is at present unknown

An experimental study of the polar forms of human leprosy would be of great interest, particularly if model infections could be established in immunologically unmanipulated animals Some previous observations indicate that infection with *Mycobacterium lepraemurium* (MLM) might provide a useful model *Stefansky* (1903) described two forms of leprosy in wild rats, the "glandular form" involving lymph nodes alone, and the "musculocutaneous form" in which the animals were cachectic showing alopecia and extensive involvement of the skin and underlying muscular tissue The opinion most wide-

ly held in the early literature was that the two forms merely represent different stages of development of the infection (*Marchoux* 1912 *Lower* 1938) Challenging this view, *Kawaguchi* (1959a) divided murine leprosy into two forms, benign and malignant, based on his observations in different strains of inbred mice Following subcutaneous inoculation, the leproma developed early in the C 57 BL/6 strain, but soon stopped growing and tended to regress spontaneously, while in the C 3 H strain, lepromata developed more slowly but became very large and ultimately caused the death of the host The course of the disease in other strains was intermediate to that seen in these two

The present paper reports on the variation in clinical course and histological findings in outbred mice infected intravenously with MLM The observations were made on mice originally infected merely to propagate bacilli and did not evolve from a planned experiment

MATERIALS AND METHODS

Infection of mice Forty 6 8 week old female white mice of the outbred NMRI strain were injected intravenously with 0 2 ml of a suspension of *Mycobacterium lepraemurium* The bacilli (Douglas strain) were kindly provided by Dr T Godal of the Armauer Hansen Research Institute Addis Ababa Ethiopia The suspension was shipped to Norway on ice and subsequently stored at 4° C it was 4 days old when used and contained approximately 2×10^8 bacilli/ml

The animals were kept in cages 10 15 mice

TABLE 1 *Frequency of Granulomas at Various Sites in Eight Mice Infected with* Mycobacterium lepraemurium

Organ	No of animals examined	No of animals with granulomas
Liver	8	8
Spleen	8	8
Lungs	7	7
Myocardium	7	7
Kidneys	6	5
Thigh muscles	8	8
Skin	4	3
Adrenals	2	2



Fig 1 Collection of macrophages infiltrating between muscle fibres Note lack of inflammatory reaction Animal No 3 Haematoxylin and eosin × 200

in each and given pellets (Norwegian Standard Stock No 1 mice and rats) and water They were observed at weekly intervals until some of them became visibly affected between 20 and 22 weeks after inoculation During the following three weeks three mice died These were not subjected to further examination On day 171 after inoculation the four most severely ill mice were sacrificed Thirty days later two mice which seemed to be completely unaffected by the infection were also killed After a further six weeks two mice one of which was in a fair clinical condition and the other cachectic were sacrificed These 8 animals were histologically examined

Histological Examination

Sections stained alternatively with haematoxylin and eosin and with the Ziehl Neelsen method (ZN) for acidfast bacilli were studied

In sections from the liver granulomas larger than approximately 60 *microns* were counted in a microscope equipped with an ocular micrometer It was not feasible to count all granulomas in the most heavily infected livers since they contained a large number of very small granulomas A lower limit of 60μ was therefore chosen for the size of granulomas to be counted

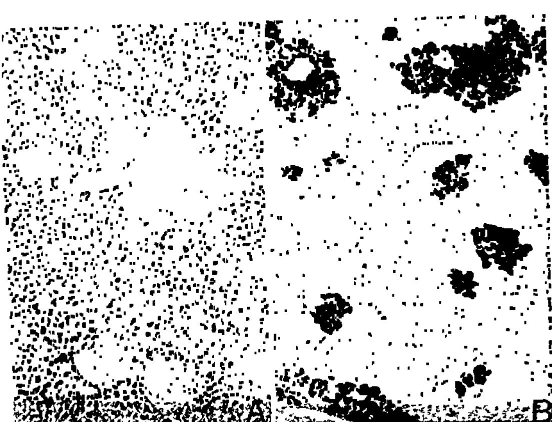


Fig 2. A Multiple, large granulomas in liver, almost without inflammatory reaction Animal No 2 Haematoxylin and eosin × 78
B. Ziehl Neelsen stained section of liver showing multiple granulomas of various size containing abundant Z-N-positive material Animal No 4 × 78

By enlargement in a projector, the liver sections were measured by planimetry and the number of granulomas calculated per square centimeter In the present context, granulomas are defined as clusters of macrophages with or without surrounding cellular reaction and containing ZN positive bacilli. Without actually counting the number of cells and the proportion of the different cell types present in the lesions, three main types of granulomas could clearly be recognized on the basis of their histological appearance those showing a *predominance of lymphocytes, those dominated by* polymorphonuclear leucocytes (PMN-leucocytes) and those in which no cellular reaction was found

RESULTS

After inoculation, the clinical condition of the animals varied considerably. Some became progressively affected and died spontaneously in a cachectic state, some were chronically ill but less affected, and some did not show clinical signs of infection up to 30 weeks after inoculation All of the animals showed definite evidence of infection at autopsy

The frequency at which the infection involved various organs in the eight animals studied, are summarized in Table 1

The lesions in thigh muscles, abdominal skin, myocardium, kidneys and adrenal glands were histologically rather uniform. groups of large, pale staining macrophages which in ZN stained sections could be shown to be loaded with bacilli, infiltrating betweeen apparently normal tissue components as shown in Figure 1 In larger lesions, central necrosis with accumulation of PMN-leucocytes was frequent Definite signs of cellular

A

B

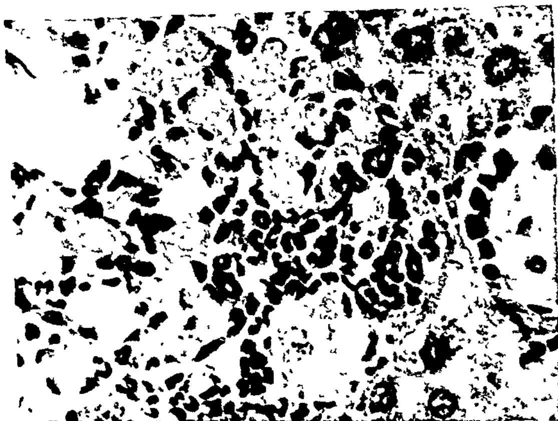


Fig 4 Small liver granuloma showing marked infiltration by PMN leucocytes and occasional plasma cells No typical, small lymphocytes are seen Animal No 2 Haematoxylin and eosin ×760

reaction surrounding such lesions were not observed

Among discrete nodules occurring in the lungs, polymorphs and mononuclear cells were occasionally encountered, but most of the pulmonary lesions appeared histologically "naked" Clinically unaffected animals did show a few lymphocytes surrounding groups of macrophages

The spleen was most extensively involved with numerous macrophages forming large nodules, some of which showed central necrosis with accumulation of polymorphs Lesions found in the spleen of clinically unaffected animals were smaller than those found in the others

In the liver the lesions were less uniform than in other sites, in some livers there was complete dissemination of the infection, the organ being packed with a large number of granulomas containing vast numbers of Ziehl-Neelsen positive bacilli (Figure 2) Two main types of granulomas were seen in such livers, one showing almost no cellular reaction (Figure 3) and another consisting essentially of larger granlomas, markedly infiltrated by polymorphs (Figure 4) These large granulomas often showed central necrosis Some small granulomas containing lymphocytes or a mixture of polymorphs, lymphocytes and plasma cells were also encountered in the severely infected livers In other livers

Fig 3 A. Liver granuloma consisting of a naked cluster of macrophages with no inflammatory reaction Animal No 2 Haematoxylin and eosin × 760

B The same granulomas stained with Ziehl Neelsen showing presence of large amounts of Z N positive material × 760

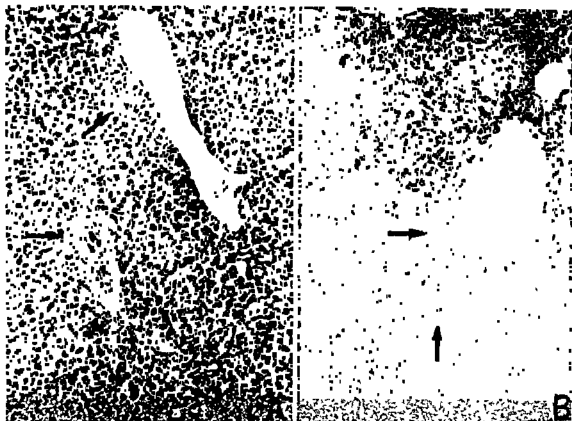


Fig 5 A Section of liver showing few and small granulomas (arrows) with marked inflammatory reaction Animal No 5 Haematoxylin and eosin × 78
B Ziehl Neelsen stained section of liver showing granulomas (arrows) with inflammatory reaction Note the scarcity of Z N-positive material in the lesions Animal No 7 × 78

the signs of infection were less striking (Figure 5) There was a paucity of granulomas, the individual lesion being small with a marked infiltration of lymphocytes (Figure 6) Macrophages in lesions surrounded by lymphocytes contained less bacilli than similar cells found in naked or polymorph-dominated granulomas

Extensive involvement of the liver correlated with poor clinical condition In contrast, the three animals which were in a good clinical condition all showed few and rather small granulomas with marked lymphocytic infiltration These findings are summarized in Table 2 The difference between animals numbers 5, 6, 7 and the others, was in fact even more pronounced than is evident from the data presented. In livers containing many large granulomas there were in addition numerous small aggregates of macrophages containing bacilli, while in those showing few large granulomas the number of small aggregates was negligible Cellular reaction was assessed in randomly selected granulomas irrespective of their size In the severely affected animals, mononuclear cells generally appeared to be plasma cells or lymphoblasts Typical, small lymphocytes only accounted for a small proportion of the cellular infiltrate,

Fig 6 A Small cluster of macrophages in liver surrounded by a large accumulation of small lymphocytes *Animal No 5 Haematoxylin* and eosin × 760
B Same granuloma stained with Ziehl Neelsen showing the presence of moderate amounts of Z-N-positive material × 760

A
B

TABLE 2 *Clinical Condition in Relation to Number of Granulomas larger than 60 Microns and Type of Cellular Reaction in the Liver of Mice infected with* Mycobacterium lepraemurium

Animal number	Days after inoculation	Clinical condition	No of granulomas per sq cm	Cellular Reaction§ † L	P	O
1	174	Poor	52	3	19	9
2	174	Poor	63	4	15	12
3	174	Poor	60	7	10	15
4	174	Poor	49	8	22	2
5	204	Excellent	2	12	0	0
6	204	Excellent	2	6	0	0
7	253	Fair	2	16	0	1
8	253	Poor	50	5	17	5

§ Number of Granulomas showing

L Predominance of lymphocytes or plasma cells
P Predominance of polymorphs, or
O No cellular reaction

† Includes data for granulomas smaller than 60 microns

while in animals numbers 5, 6 and 7 they were the main type of inflammatory cells

COMMENT

In outbred mice, progressive generalized infection occurs regularly after intravenous injection of MLM Following subcutaneous inoculation, infection may be resisted for a long time in some animals (*Fite* 1940) Abortive infection may occasionally also occur after intraperitoneal injection of MLM (*Sellards* & *Pinkerton* 1938) This indicates that although mice in general easily become infected with MLM, there exist individual differences in their susceptibility

Several strains of inbred mice have been reported to be susceptible to infection by MLM by various routes of inoculation (*Sellards* & *Pinkerton 1938*, *Krakower* & *Gonzales* 1940) *Kawaguchi* (1959a) infected mice of several inbred strains subcutaneously and classified murine leprosy into two polar forms, malignant and benign, based on the clinical appearance of the lepromas In the benign form, the leproma soon stopped growing and tended to regress spontaneously while in the malignant form, the leproma continued to grow until fatal dissemination occurred In the individual strain one clinical form seemed to dominate, indicating that genetic factors of the host influence resistance to infection

In human leprosy there is a clear relationship between the CMI of the host, the histological appearance of the lesions, and the clinical course Two polar types of histological reactions are seen In the tuberculoid type the lesions consist of epitheloid cells surrounded by an adundance of lymphocytes and they do not contain bacilli In the lepromatous type there is proliferation of macrophages loaded with bacilli, but no surrounding lymphocytes are seen The presence of lymphocytes in an inflammatory reaction generally indicates that CMI is involved (*Suter* & *Ramseier* 1964) The clinical course of human leprosy seems to be greatly influenced by the CMI of the host, across the leprosy spectrum there is an inverse relationship between the number of bacilli in the macrophages and the degree of lymphocytic infiltration in the lesions This is parallelled by the reactivity of peripheral lymphocytes *in vitro* to antigens derived from *M leprae* as shown in experiments involving lymphocyte transformation (*Godal et al* 1971, *Han et al* 1971)

As regards murine leprosy limited data are available concerning the correlation between histological picture and clinical course According to indirect citations, *Kawaguchi* (1959b) who studied inbred strains of mice

observed that, in the malignant form, the lesions contained almost no epitheloid cells and did not show infiltration by monocytes or lymphocytes Marked lymphocytic infiltration was seen in the benign form in which the lesions contained many epitheloid cells as well As regards outbred mice, no attempts have been made to correlate variation in susceptibility with different histological pictures with a view to revealing a possible spectrum resembling that of human leprosy

In the present study, the relative proportion of lymphocytes in the lesions was used as an indicator of the CMI-response of the host against MLM, in an attempt to uncover differences in the CMI response, several organs were examined histologically In the majority of organs the lesions were uniform, consisting of naked aggregates of MLM containing macrophages The number and size of granulomas found in the liver and spleen varied in the animals (cf Table II) Since cellular reaction was impossible to assess in the spleen, but was easily demonstrable in the liver, this organ remained the only one suitable for the purpose of the present investigation

After intravenous inoculation, the bacilli would be expected to lodge mainly in organs with large phagocytosing capacity, i e liver, spleen and lungs which would harbour the primary lesions Lesions at other sites presumably develop as a result of a secondary dissemination of bacilli As shown by *Ptak et al* (1970), MLM infection in mice induces a general depression of the CMI response At the stage when secondary dissemination occurs, it is conceivable that either competent lymphocytes are alredy engaged (*Mackaness* 1971b) or the CMI response in general has been markedly reduced This would explain why, in the same animal, prominent lymphocytic infiltration could be seen in the liver but was completely lacking in other sites.

Comparing severely affected and clinically virtually unaffected mice, marked histological differences were demonstrated in the liver Since the same volume of bacillary suspension was injected intravenously into all animals, it is unlikely that differences in the number of bacilli received by each animal could explain the present findings *Fite* (1940) reported that MLM-infection in mice proceeded more slowly after subcutaneous than after intravenous inoculation If we consider that some of our mice, i e the least affected ones, might have received part of the injected volume subcutaneously it might explain the present findings To do so one would have to assume that all, or nearly all, bacilli were injected s c which is not a very likely possibility Animals receiving as much as half of the volume intravenously would only lag one generation time (10 12 days) behind animals receiving a full dose Since the least affected animals were sacrificed as many as 30 days after the others, the possible error introduced by injecting even 90 per cent of the suspension s c is probably insignificant

On the assumption that all mice received the bacilli i v the present observations demonstrate that mice vary in their susceptibility to infection by MLM Clinically, some animals remained unaffected when the majority were either very sick or had already succumbed Corresponding to the severity of the infection, marked histological differences were seen in the liver Unaffected animals had few and small granulomas which contained relatively few bacilli and were surrounded by a border of small lymphocytes In contrast, the liver granulomas in severely affected mice were composed of large aggregates of macrophages heavily loaded with bacilli The majority of these lesions did not contain lymphocytes

It is concluded on the basis of the present findings that some animals possess a stronger ability than others to mount a CMI reaction against the bacilli

In MLM infected mice, a spectrum apparently exists which bears some resemblance to that observed in human leprosy Clearly the differences observed were less marked than the difference between LL and TT In particular, an equivalent to the tuberculoid type was not encountered The reasons for this may partly be that the dose injected

was so large that even animals showing relatively efficient CMI response failed to prevent dissemination of the infection

The full establishment of a spectrum would further expand the usefulness of murine leprosy as an experimental model By varying the dose and the route of inoculation and by using genetically homogenous strains, the spectrum may be further developed Experiments along these lines are at present in progress

This work was supported by grants from *Anders Jahre's Fund for the Promotion of Science* and *The Norwegian Research Council for Science and the Humanities*

REFERENCES

1 *Fite, G L* II Leprosy The pathology of experimental rat leprosy Nat Inst Health Bull *173* 45–79, 1940

2 *Godal, T , Myklestad, Berit, Samuel Dorothy R & Myrvang B* Characterization of the cellular immune defect in lepromatous leprosy A specific lack of circulating Mycobacterium leprae reactive lymphocytes Clin exp Immunol *9* 821 831 1971

3 *Han, S H , Weiser, R S & Lin, Y C* Transformation of leprous lymphocytes by leprolin tuberculin and phytohemagglutinin Int J Leprosy *39* 789–795, 1971

4 *Kawaguchi Y* Classification of murine leprosy Jap J Exp Med *29* 651 663 1959 a

5 *Kawaguchi, Y* Classification of experimental mouse leprosy La Lepro *28* 378–388 1959 b Cited in Nishimura S Murine Leprosy In Infektionskrankheiten und ihre Erreger Band 4, Meissner, Gertrud and Schmiedel A J (Eds) Mykobakterien und Mykobakterielle Krankheiten Teil IX Human and Murine Leprosy Gustav Fischer Verlag, Jena 1967 p 356

6 *Krakower, C & Gonzales, L M* Mouse leprosy Arch Path *30* 308 329, 1940

7 *Lowe, J* Rat leprosy A critical review of the litterature Int J Leprosy *5* 311 328 and 463–481, 1937

8 *Mackaness, G B* Resistance to intracellular infection J Infect Dis *123* 439–445, 1971a.

9 *Mackaness, G B* Delayed hypersensitivity and the mechanism of cellular resistance to infection In Amos, (Ed) Progress in Immunology Academic Press New York and London 1971b , p 413–424

10 *Ptak, W , Gaugas, J M , Rees, R J W & Allison, A C* Immune response in mice with murine leprosy Clin exp Immunol *6* 117–124, 1970

11 *Rees, R J W , Chatterjee, K R , Pepys, J & Tee, R D* Antigen studies of other fungi and *Mycobacterium leprae* Some immunological aspects of leprosy Am Rev Resp Dis. *92*

12 [illegible] system Int. J Leprosy *34* 255–273, 1966

13 *Sellards, A W & Pinkerton H* The behaviour of murine and human leprosy in foreign hosts. Am J Pathol *15* 421–434, 1938

14 *Stefansky, W K* Eine lepraahnliche Erkrankung der Haut und Lymphdrusen bei Wanderratten Centralbl f Bakt *33* 481–487, 1903

15 *Suter, E & Ramseier, H* Cellular reactions in infection Adv Immunol *4* 117–173, 1964

16 *Turk J L & Bryceson A D M* Immunological phenomena in leprosy and related diseases Adv Immunol *13* 209 266 1971

Acta path. microbiol scand Section A 81, 411–424, 1973

DISTRIBUTION OF PITUITARY CELL TYPES IN RELATION TO THE HISTOLOGY OF THE PROSTATE IN ELDERLY MEN

An Analysis in an Autopsy Series

OLAV A. HAUGEN

Ullevål Hospital, Department of Pathology, University of Oslo, Oslo, Norway
Head Kristen Arnesen, M D

The distribution of cell types in the pituitary gland was studied in a consecutive autopsy series of men over 40 years of age Among 166 individuals, not previously subjected to prostatic surgery or oestrogen treatment, the proportion of PAS positive cells was higher in men with hyperplasia or neoplasia of the prostate than among individuals showing a histologically normal prostate PAS positive cell counts were negatively influenced by several factors related to wasting disease, while acidophil cells apparently increased in conditions of acute and severe stress Using multiple regression analysis, benign prostatic hyperplasia was selected as the only one among histological diagnoses which significantly reduced the variability of PAS positive cell proportion, even when the negative influence of other factors had been accounted for PAS positive cell counts did not effectively discriminate between the various forms of abnormal prostatic growth encountered The data presented indirectly give support to the concept of some form of endocrine derangement in the ageing male

Clinical studies suggest that benign hyperplasia and carcinoma of the prostate are hormone related conditions (*Huggins* & *Hodges* 1941 *Moore* 1947) The hormone dependency of the normal prostate is well documented and involves both testicular androgens and pituitary hormones (for references, see *Ofner* 1968)

Pituitary hormone studies in patients with prostatic hypertrophy and carcinoma are limited However, observations of increased urinary excretion of prolactin (*Asano* 1965) and decreased luteinizing hormone (LH) reserve (*Geller et al* 1970) in patients with benign hypertrophy or carcinoma of the prostate may indicate abnormal pituitary function in these conditions.

On the assumption that an altered hormonal state might also be reflected in the morphological appearence and distribution of pituitary cells, attempts have been made to correlate certain cell classes to abnormal growth of the prostate (*Jones* 1939, *Mellgren* 1945, *Sommers* 1957, *Russfield* & *Byrnes* 1958, *Dekker* & *Russfield* 1963, *Koppel et al* 1967) The results from these studies are conflicting However, *Sommers* (1957) examined a large number of glands and found acidophil

Received 19 1 73 Accepted 19 3 73

Requests for reprints should be addressed to *Department of Pathology, Ullevål Hospital, Oslo 1, Norway*

cell hyperplasia and small pituitary adenomas to be unusually frequent in patients with carcinoma of the prostate

Haugen (1973) observed increased pituitary weight among men with benign hyperplasia and carcinoma of the prostate The present report describes the distribution of pituitary cell types in the same series of men The distribution of pituitary cell types was analysed in relation to prostatic histology and several other factors Multiple regression analysis was applied in order to assess the relative importance of various factors and to adjust for confounding variables

The present investigation is part of a larger study of the relationship between pathological growth of the prostate and the morphology of the pituitary gland, the adrenal glands and the testes The histological findings in the prostate in this series have been reported previously (*Harbitz* & *Haugen* 1972)

MATERIAL AND METHODS

The pituitary glands and the prostates from 206 consecutive autopsies of men over 40 years of age were collected during a 3 month period 1967–1968 Forty patients were excluded from the main analysis for the following reasons previous prostatic surgery (24 patients), oestrogen treatment for clinically manifest prostatic carcinoma (6 patients), secondary tumour invasion of the prostate or testes (4 patients) and seminoma of the testis (1 patient) In three instances the slides were unsuitable for cell counting, one block was lost and in one patient with a large pituitary adenoma cell counting was not performed The analysis was finally based on 166 patients none of whom showed clinical evidence of prostatic carcinoma Apart from seven patients with clinically manifest diabetes mellitus none were known to suffer from other endocrinological disorders

The pituitary glands were removed and fixed in Helly s fluid (Zenker Formol) as described previously (*Haugen* 1973) The fixed glands were bisected through the horizontal plane into equal halves and washed in running tap water for 24 hours before dehydration and embedding With the microtome set at 5 microns one section was cut from each half as close as possible to the cut surface The sections were placed on slides with their longest axis parallel to the long side of the slide Staining was performed with periodic acid Schiff (PAS) Orange G (*Pearse* 1950)

Classification of Pituitary Cells

Three main types of cells were distinguished

1 *Orange G positive cells*, i e cells which are densely packed with granules giving a bright yellow colour with Orange G
2 *PAS positive cells*, i e cells showing varying amounts of intracytoplasmic PAS positive material, either diffusely distributed or in distinctly formed granules
3 *Chromophobe cells*, i e cells which do not contain PAS positive material or distinctly formed granules with affinity to Orange G Such cells were of varying size, but no distinction was made between large and small chromophobe cells

Differential Counts

The counting procedure was modified from the methods described by *Rasmussen* & *Herrick* (1922) and *Mellgren* (1945) and was performed with a microscope equipped with a squared ocular net measuring 5 × 5 mm. The counts were made under magnification × 1250 (oil immersion)

In order not to overestimate the frequency of

constantly moving the micrometer nuclei within the plane of the section clearly increasing and de

vertical and horizontal direction towards the opposite end of the section, nucleated cells were counted in every 15th square in every 15th row of squares over the whole section In this way, the total number of cells counted varied from 195–619 the mean number of cells counted being 363 The counts were made without any knowledge of the clinical data

Histological Classification of the Prostate

The procedures of dissection and histological examination of the prostate have been described previously (*Harbitz* & *Haugen* 1972) The presence of benign nodular hyperplasia (BNH), carcinoma (C) atypical glandular proliferation (AGP) or diffuse atrophy (DA) was noted for each prostate The presence of AGP in prostates showing carcinoma was not recorded A histologically normal prostate (N) showed none of the characteristics mentioned above

The histological findings in the prostates of 166 patients included in the main analysis appear from Table 1

Clinical Data

Clinical data were recorded from the clinical notes and prepared for the computer analysis.

stical Methods

odified Student s t test accounting for unequal ınces and numbers of individuals were used for ng differences between arithmetic means and testing differences between slopes of regression ; (*Snedecor* & *Cochran* 1967) n_A and n_B being number of observations in the groups to be pared, p values were based on the least of n_A 1 n_B 1 (for means) or n_A 2 and n_B 2 (for es) degrees of freedom P values below 0 05 e regarded statistically significant

Iultiple regression analysis was applied as preısly described (*Haugen* & *Harbitz* 1972, *Hau* 1973) using the proportions of Orange G

to X_{12}) or continuous (labelled X_{13} to X_{15}) e treated as *explanatory* (independent) varia s

tology of the Prostate

X Benign nodular hyperplasia (BNH)

use of Death

X_6 Cardiovascular disease*

X_7 Malignant tumour

ration of Final Illness

X_8 1 7 days

X_9 > 7 days

her

X_{10} Steroid hormone treatment§

X_{11} Diabetes mellitus

X_{12} Liver cirrhosis

X_{13} Age

X_{14} Body weight

X_{15} Body length

tially forward stepwise regression analysis was d until all explanatory variables which were partially significant at the 5 per cent level at each step, were included Thereafter, the selected variables together with all groups of prostatic histology (X_2 to X_5) were included in the full multiple regression analysis. Regression coefficients were calculated according to the method of least squares

The analysis was based on a standard program for multiple regression analysis (NRSR) developed at The Norwegian Computing Center, Oslo, and was conducted on a Univac 1108 computer

The reproducibility of the method applied for differential counts of pituitary cells was tested by duplicate counts in 13 randomly selected cases These sections were drawn by another member of the staff and mixed with sections which had not *been counted previously During all counts the* identification number of the sections were covered with tape and there was a time lag of several months between first and second count The results have been plotted in Fig 1 The *method error* was computed from the usual formula $\sqrt{\frac{\Sigma D^2}{2n}}$, where D denotes the difference between duplicates and n the number of pairs compared The method error, expressed as per cent of all cells counted was as follows

Orange G positive cells	8 8 %
PAS positive cells	12 8 %
Chromophobe cells	14 5 %

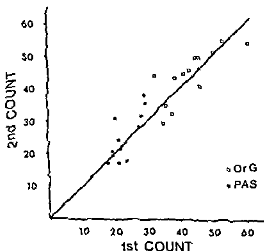


Fig 1 Scatter plot showing duplicate counts of Orange G positive (OrG) and PAS positive (PAS) cells in per cent of total cell counts

* Includes death from myocardial infarction (48 cases) cerebrovascular and peripheral vascular disease (15 + 7 cases) rheumatic valvular disease 4 cases) miscellaneous cardiovascular disorders (10 cases)

§ Includes treatment with corticosteroids (7 cases) anabolic steroids (nortestosterone) (5 cases), or both (11 cases)

TABLE 1 *Histology of the Prostate by Age in 166 Patients**

Age	N	DA	BNH	C+BNH	C	AGP+BNH	AGP
40–49	1	1	1	0	0	0	0
50–59	10	5	10	3	1	5	0
60 69	7	1	20	15	5	6	1
70–79	0	1	28	21	0	5	1
80 +	0	0	8	8	0	2	0
All	18	8	67	47	6	18	2

* N = normal histology, DA = diffuse atrophy, BNH = benign nodular hyperplasia, C = carcinoma AGP = atypical glandular proliferation

RESULTS

The distribution of the proportions of Orange G positive cells, PAS positive cells and chromophobe cells appears from Fig 2

A slight decrease in the proportion of Orange G positive cells and a slight increase in PAS positive cells with advancing age was seen (Table 2) However, the association with age was not statistically significant for any type of cells (Table 8)

Table 3 presents the proportions of pituitary cells in relation to body weight Low body weight was apparently associated with low number of PAS positive cells and the number of such cells increased with increasing body weight The reverse situation was observed for chromophobe cells The association of these cells with body weight was weak, but the correlation coefficients were statistically significant (Table 8)

No association was demonstrated between any cell type and body length (Table 4)

Table 5 presents the proportion of pituitary cells in relation to cause of death duration of final illness, steroid hormone treatment, diabetes mellitus and liver cirrhosis High values of Orange G positive cells were observed in patients dying after short term illness, but the mean value was not statistically different from that observed in cases of sudden deaths ($p > 0\ 05$) High values also appeared in patients treated with steroid hormones In patients dying from malignant tumours, the proportion of PAS positive cells was significantly lower than that observed in patients dying from cardiovascular disease ($p < 0\ 01$), but not significantly different from that observed in patients dying from other conditions ($p > 0\ 10$) Low mean values for PAS positive cells were also seen in relation to protracted final illness and in patients treated with steroid hormones

Obviously, some of the factors related to low mean values of PAS positive cell counts are likely to be interrelated Steroid hormone treatment had preferably been given to patients with malignant tumours (17/23), who often died after protracted illness Furthermore, low body weight, which was apparently associated with low proportions of PAS-positive cells (Table 3) is also linked to protracted final illness Among patients weighing less than 60 kg, more than 70 per cent had protracted final illness and about 40 per cent died from malignant tumours A comparison of the mean values of PAS positive cells in cases of sudden deaths in the weight classes below 60 kg (25 6 per cent PAS positive cells, 8 patients) and similar cases in the weight class above 80 kg (27 9 per cent PAS-positive cells, 9 patients) revealed that the difference was not statistically significant ($p > 0\ 50$)

The proportion of pituitary cell types in relation to the histology of the prostate appears from Table 6 No significant differences in the number of Orange G positive cells in the histological groups could be demonstrated.

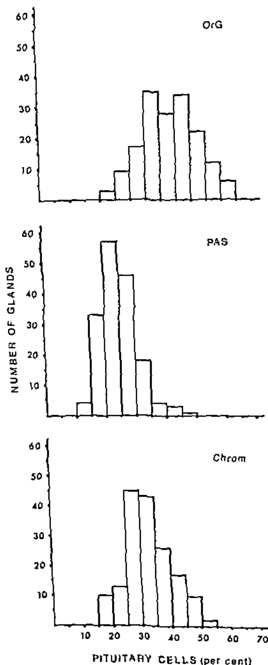


Fig 2 Distribution of Orange G positive (OrG) PAS positive (PAS) and chromophobe (Chrom) cells in per cent of total cell counts among 166 men over 40 years of age

The low values of PAS positive cells in patients with DA of the prostate probably is related to the fact that six patients died from malignant tumours and they all were related

a ... p

hyperplasia or neoplasia of the prostate ($p < 0.005$)

In patients previously subjected to prostatic surgery *for benign prostatic hyperplasia* the proportion of PAS positive cells was also high (Table 7) The numbers of PAS positive cells in the few patients who had received treatment with oestrogen were much lower than those otherwise to be expected according to their high age (Table 7)

From the scatter diagrams (Fig 3) it is apparent that the individual values of Orange G positive cells and of PAS positive cells varied within all histological groups and at all age levels The corresponding regression lines are presented in Fig 4 The regression lines did not differ significantly from each other but the regression line for Orange G positive cells on age in patients with BNH ($b = -0.341$) *(Fig 4 A) was significantly different* from zero ($p < 0.01$)

In the course of the multiple regression analysis simple correlation analysis was also performed The correlation coefficients are presented in Table 8 Orange G-positive cells showed a significant correlation to short term illness only A positive and statistically significant relationship was demonstrated between the proportion of PAS positive cells and the occurrence of BNH (X_2), death from cardiovascular disease (X_6) and body weight (X_{14}) PAS-positive cells showed a negative correlation with the presence of DA of the prostate (X_5) death from malignant tumours (X_7) long term illness (X_9) and treatment with steroid hormones (X_{10}) Chromophobe cells showed a positive correlation with death from malignant tumours (X_7) and protracted final illness while a negative relationship to short term illness (X_8) and body weight (X_{14}) was demonstrated

27*

TABLE 2 *Proportions of Pituitary Cell Types (per cent) by Age*

Age	No patients	OrG		PAS		Chrom	
		Mean	S D	Mean	S D	Mean	S D
40–49	3	53.1	5.7	19.0	5.5	27.9	9.8
50–59	34	43.8	9.4	22.9	4.3	33.2	8.7
60–69	55	43.4	8.8	25.0	6.1	31.6	8.2
70–79	56	43.5	9.6	24.7	6.7	31.8	6.5
80 +	18	39.8	7.6	25.8	5.8	34.4	5.9
All	166	43.3	9.1	24.4	6.0	32.3	7.5

S D Standard deviation

TABLE 3 *Proportions of Pituitary Cell Types (per cent) by Body Weight*

Body weight (kg)	No patients	OrG		PAS		Chrom	
		Mean	S D	Mean	S D	Mean	S D
< 50	25	43.3	9.6	23.0	4.2	33.8	9.8
50–59	45	43.4	8.2	23.5	5.6	33.1	6.7
60–69	41	43.0	9.5	25.0	6.9	32.0	7.0
70–79	35	43.1	9.2	25.6	5.5	31.3	7.3
80–89	14	43.5	10.1	24.6	6.7	31.9	7.7
90 +	6	46.1	9.9	26.7	7.2	27.2	7.1
All	166	43.3	9.1	24.4	6.0	32.3	7.5

S D Standard deviation

TABLE 4 *Proportions of Pituitary Cell Types (per cent) by Body Length*

Body length (cm)	No patients	OrG		PAS		Chrom	
		Mean	S D	Mean	S D	Mean	S D
< 160	4	47.1	9.7	26.7	5.4	26.2	9.7
160–164	16	42.5	7.9	23.5	5.4	34.0	6.7
165–169	35	44.3	9.3	22.8	6.6	32.4	7.9
170–174	54	42.9	7.8	24.9	6.0	32.3	7.4
175–179	37	43.3	10.6	24.6	6.5	32.1	7.5
180–184	13	45.4	11.2	25.1	7.3	29.5	7.3
185 +	7	39.0	7.0	24.8	3.2	36.4	7.6
All	166	43.3	9.1	24.4	6.0	32.3	7.5

S D Standard deviation

Multiple linear Regression Analysis

If the stepwise procedure was performed with the total set of explanatory variables (X_2–X_{15}), short term illness (X_8) was the only factor which significantly reduced the variance of Orange G positive cells PAS-positive cells were significantly influenced by death from malignant tumours (X_7) and the

TABLE 5 *Proportions of Pituitary Cell Types (per cent) in Relation to Cause of Death, Duration of Final Illness, Steroid Hormone Treatment, Diabetes Mellitus and Liver Cirrhosis among 166 Patients*

	No patients	OrG		PAS		Chrom	
		Mean	S D	Mean	S D	Mean	S D
Cause of death							
Cardiovascular disease	84	43.1	8.6	25.6	5.9	31.3	7.4
Malignant tumour	43	42.7	10.0	22.5	6.0	34.8	7.9
Other conditions	39	44.4	9.1	24.2	5.6	31.4	6.9
Duration of final illness							
< 1 day	36	42.6	9.6	26.3	6.5	31.1	6.9
1–7 days	32	46.3	7.6	24.8	4.6	29.0	6.2
> 7 days	98	42.6	9.1	23.6	6.0	33.8	7.8
Steroid hormone treatment*	23	45.2	9.6	21.6	4.2	33.1	8.4
Diabetes mellitus	7	42.4	6.9	25.8	6.3	31.9	3.8
Liver cirrhosis	5	40.8	12.8	26.1	8.3	33.0	8.0

* Other than oestrogenic hormones
S D Standard deviation

TABLE 6 *Pituitary Cell Types (per cent) by Histology of the Prostate**

Histology of the prostate	No patients	OrG		PAS		Chrom	
		Mean	S D	Mean	S D	Mean	S D
N	18	45.5	9.1	21.4	4.1	33.1	8.7
DA	8	45.9	13.7	19.8	3.7	34.3	11.4
BNH	67	43.6	9.5	25.0	5.8	31.4	7.2
C + BNH	47	42.2	8.5	25.0	6.6	32.8	7.2
C	6	42.5	4.2	27.0	4.7	30.5	8.2
AGP + BNH	18	41.3	7.6	24.8	6.3	33.9	5.8
AGP	2	49.7	4.6	26.3	5.4	24.0	10.0
All	*166*	43.3	9.1	24.4	6.0	32.3	7.5

S D Standard deviation
* For abbreviations, see Table 1

TABLE 7 *Proportions of Pituitary Cell Types (per cent) among Patients previously Subjected to Prostatic Surgery or Oestrogen Treatment*

Category	No patients	Mean age	OrG		PAS		Chrom	
			Mean	S D	Mean	S D	Mean	S D
Prostatectomy	23	73.6	40.2	9.2	27.3	6.1	32.4	5.8
Oestrogen treatment	6	71.3	39.2	6.0	21.8	6.9	38.9	4.3

S D Standard deviation

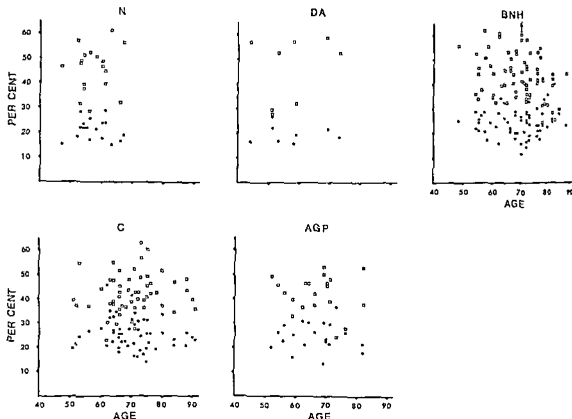


Fig 3 Scatter diagrams showing proportions of Orange G positive cells (open squares) and PAS positive cells (black circles) in relation to age of men with histologically normal prostate (N), diffuse atrophy (DA), benign nodular hyperplasia alone (BNH), carcinoma with and without BNH (C) and atypical glandular proliferation with or without BNH (AGP)

presence of BNH (X_2), while the variation of chromophobe cells was significantly reduced by long-term illness (X_9) only

The full regression analysis was run with all the variables for prostatic histology (X_2–X_5) together with the variables selected at the stepwise procedure The results appear from Table 9 With regard to Orange G-positive cells, none of the regression coefficients for prostatic histology were statistically significant, nor did they differ significantly from each other ($F = 0\,683$, $f_1 = 4$ $f_2 = 160$, $p > 0\,25$) Table 9 a) In the analysis of PAS-positive cells (Table 9 b), BNH showed the highest regression coefficient among the histological diagnoses, but was no longer statistically significant The regression coefficients for all groups of prostatic histology did not differ significantly from each other ($F = 1\,749$, $f_1 = 4$, $f_2 = 160$, $0\,025 < p = 0\,10$)

Concerning the chromophobe cells, the regression coefficients for all groups of prostatic histology did not differ significantly from each other ($F = 0\,082$, $f_1 = 4$, $f_2 = 160$, $p > 0\,25$) (Table 9 c)

The total set of variables included in the full regression analysis had explanatory values of about 0 04, 0 077 and 0 059 respectively This implies that the factors tested in this model have negligible effects upon the degree of explanation for the variation in proportions of pituitary cell types

COMMENT

The heterogeneity of most autopsy series makes comparison of data between different series and even within the same series difficult Statistically, the ideal approach would

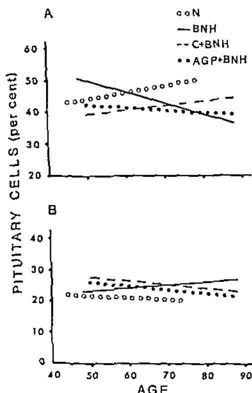


Fig 4 Regression lines for proportions of Orange G positive cells (A) and PAS positive cells (B) on age in different groups of prostatic histology Normal histology (N) Benign nodular hyperplasia alone (BNH), carcinoma with hyperplasia (C + BNH) and atypical glandular proliferation with BNH (AGP + BNH)

be to study a large number of individuals to mask the effect of all confounding variables As a substitute, multiple regression analysis was applied in the present study in an attempt to uncover and account for the effect(s) of factors not related to the histology of the prostate The explanatory value of the factors selected however, was low and interpretation of the results will therefore not be restricted to this model

For screening purposes on a large scale differential counts must be relatively rapid and the method must be shown to give reproducible results Since it has been shown that the distribution of the various cell types varies less in horisontal than in sagittal sections at different levels of the adenohypophysis (*Rasmussen* 1929, *Golden* 1959) counts were performed on horizontal sections only In the present series, the number of cells counted in each case was considerably less than that advocated by others (*Rasmussen* 1929, *Sommers* 1958), yet for the purpose of this study reproducibility of the counts was within acceptable limits

For a number of reasons, the simple classification of cells in the adenohypophysis as acidophil, basophil and chromophobe may appear inadequate Although specific hormones have been related to distinct cells with variable morphology, staining properties, and location, no single staining method is available which specifically discriminates between cells with different hormone production Acidophil (Orange G positive) cells produce somatotrophic hormone (STH) and prolactin (LTH) (*Herlant* & *Pasteels* 1967, *Pasteels et al* 1972) Follicle stimulating hormone (FSH), LH (or ICSH), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH) and melanocyte stimulating hormone (MSH) are produced by cells which give a PAS positive reaction (*Purves* 1966, *Herlant* & *Pasteels* 1967, *Bain* & *Ezrin* 1970) Chromophobe cells probably represent degranulated acidophil or basophil cells (*Purves* 1961) some of which may possess high secretory activity (*Herlant* & *Pasteels* 1967)

In accordance with other reports (*Ezrin et al* 1958, *Sommers* 1958, *Ezrin* & *Murray* 1963) acidophil cells were the most numerous cell type observed, the proportion of which remained fairly constant until high age This observation also coincides with reports according to which the pituitary STH activity is unrelated to age (*Russfield* 1960) The increase of acidophil cells in relation to short term illness may be due to chance occurrence However, *Shanklin* (1956) observed increased acidophil cell proportions in patients dying from severe burns, and stressful stimuli increase the secretion of growth hormone (*Landon* & *Greenwood* 1969) In agreement with observations by *Ezrin* & *Murray* (1963), acidophil cells also appeared to be increased

TABLE 8 *Relationship between Different Pituitary Cell Types and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variables	Dependent variables (X_1)		
	OrG	PAS	Chrom
	Correlation coefficients ($n_1 = 166$)		
Histology of the prostate§			
X_2 BNH (n = 132)	−0 113	0 185*	−0 008
X_3 AGP (n = 20)	−0 049	0 033	0 031
X_4 C (n = 53)	−0 078	0 096	0 024
X_5 DA (n = 8)	0 064	−0 177*	0 061
Cause of death			
X_6 Cardiovascular disease (n = 84)	−0 020	0 188*	−0 121
X_7 Malignant tumour (n = 43)	−0 041	−0 194*	0 202*
Duration of final illness			
X_8 1–7 days (n = 32)	0 160*	0 034	−0 213*
X_9 > 7 days (n − 98)	−0 092	−0 166*	0 238*
Other			
X_{10} Steroid hormone treatment (n = 23)	0 084	−0 189*	0 046
X_{11} Diabetes mellitus (n = 7)	−0 020	0 046	−0 010
X_{12} Liver cirrhosis (n = 5)	−0 049	0 050	0 018
X_{13} Age (n = 166)	−0 121	0 116	0 056
X_{14} Body weight (n = 166)	0 007	0 191*	−0 162*
X_{15} *Body length (n = 166)*	−0 057	0 055	0 029

§ [illegible]
n_1 [illegible]
n [illegible] or bivariate variables) or [illegible]
* Significant at the 5 per cent level

after steroid hormone treatment Stress and several pharmacological agents, including steroid hormones, may increase secretion of prolactin (*Meites* 1972) Morphologically, the increase of acidophil cells to appear in conditions of acute stress and after steroid hormone treatment may indicate altered synthesis or secretion both of STH and prolactin

The mean values of PAS-positive cells were significantly higher after the age of 60 years compared with younger individuals ($p < 0 05$) The association with age as a whole, however, was rather weak, and even among individuals in the higher age classes there was a marked variation in the proportion of these cells The present findings agree with similar observations by *Floderus* (1944), *Pearse* (1953) and *Swanson* & *Ezrin* (1960) Age-associated increase of PAS-positive cells in elderly men would be in consistence with reports on increase of pituitary gonadotropins (*Ryan* 1962), increased plasma levels of LH after the age of 50 years (*Schalch et al* 1968, *Nissen Meyer* 1972) and increased urinary excretion of gonadotrophins with age (*Johnsen* 1959) Recent studies, however, have suggested that the increased excretion of gonadotropins are mainly due to a significant increase of FSH, with a subsequent rise in the FSH/LH-ratio (*Christiansen* 1972) The metabolic pattern of testosterone in male senescence is suggestive of hypogonadism (*Vermeulen et al* 1972), and morphologically the present findings would be in consistence with some form of testicular failure leading to pituitary hyperactivity with advancing age

The low PAS counts observed in relation to

TABLE 9 *Pituitary Cell Types and the Histology of the Prostate § Full Regression Analysis*

a Explanatory variables	OrG positive cells (X_1 n = 166) Partial correlation coefficient	Partial regression coefficient	Significant at level
X_{33} 1–7 days (n = 32)	0.149	3.48	0.058
X_2 BNH (n = 132)	−0.069	−1.72	0.384
X_4 C (n = 53)	−0.055	−1.12	0.488
X_3 AGP (n = 20)	−0.046	−1.32	0.558
X_5 DA (n = 8)	0.031	1.44	0.700
Multiple correlation coefficient (R)	0.205		

b Explanatory variables	PAS positive cells (X_{11} n = 166) Partial correlation coefficient	Partial regress on coefficient	Significant at level
X_7 Malignant tumour (n = 43)	−0.172	−2.38	0.028
X_2 BNH (n = 132)	0.118	1.89	0.135
X_4 C (n = 53)	0.086	1.13	0.277
X_5 DA (n 8)	−0.053	−1.65	0.500
X_3 AGP (n = 20)	0.036	0.67	0.645
Multiple correlation coefficient (R)	0.279		

c Explanatory variables	Chromophobe cells (X_1 n = 166) Partial correlation coefficient	Partial regression coefficient	Significant at level
X_9 > 7 days (n = 98)	0.228	3.59	0.004
X_3 AGP (n = 20)	0.041	0.96	0.605
X_5 DA (n = 8)	0.025	0.97	0.754
X_2 BNH (n 132)	0.011	0.23	0.889
X_6 C (n = 53)	0.008	0.14	0.917
Multiple correlation coefficient (R)	0.243		

§ For abbreviations of histological diagnoses see Table 1

a number of conditions related to wasting disease also are in keeping with observations on low gonadotropins in malnourished individuals (*Zubiran & Gomez Mont* 1953, *Russfield & Sommers* 1963) A subtype of basophil cells believed to produce gonadotropins, were claimed by *Ezrin et al* (1958) to be reduced in wasting disease The interpretation of these cells as gonadotrophic cells have been supported (*Herlant & Pasteels* 1967) and disputed (*Halmi & McCormick* 1969)

The significance of the positive association demonstrated between PAS-positive cells and hyperplasia or neoplasia of the prostate in the present series is uncertain As the number of patients with a histologically normal prostate was small and did not exactly match the test groups, the observation may have occurred

by chance However, in the same series of men it was previously shown that the pituitary weight was maintained to a larger extent with advancing age in men with hyperplasia or neoplasia of the prostate than among subjects with a histologically normal prostate (*Haugen* 1973) Limited data available (*Geller et al* 1970) also suggest that pituitary function in men with hypertrophy or carcinoma of the prostate may be abnormal Yet, since abnormal growth of the prostate is strongly age related (*Harbitz* & *Haugen* 1972) and reliable controls are difficult to establish, morphological and functional alterations of the endocrine glands may be phenomena common to ageing males, rather than bearing any specific relationship to the pathogenesis of prostatic disorders

By design, the present study differs markedly from previous investigations on pituitary cell distribution in relation to prostatic hyperplasia or neoplasia, which makes comparison to other reports difficult *Jones* (1939), *Mellgren* (1945) and *Russfield* & *Byrnes* (1958) were unable to show any difference in pituitary cell distribution between men with prostatic hypertrophy compared with subjects showing prostates of normal size Clearly, these observations are not directly relevant as to possible differences between histologically different lesions Based on autopsy file materials, *Sommers* (1957) reported acidophil cell hyperplasia to be unusually frequent in relation to prostatic carcinoma, most of which were clinically manifest This observation however, is not well documented in the counts reported His data do reveal, on the other hand significantly higher counts of basophil cells in patients with BNH than among the controls The reported low number of basophil cell in patients with prostatic carcinoma could possibly be explained by the fact that several of his patients had multiple primary cancer or widespred metastatic lesions or they had been treated with oestrogens These factors would all tend to decrease the proportion of basophil cells The pituitary content of gonadotropins in patients treated with oestrogens have been found to be markedly reduced or absent (*Russfield 1960, Dekker & Russfield 1963*) Among the few patients in the present series who had received oestrogens, PAS positive cell counts were also unexpectedly low and close to those in patients with a histologically normal prostate

In conclusion, the present findings suggest that the proportion of PAS positive cells may be higher in men with hyperplasia or neoplasia of the prostate than among subjects with a histologically normal prostate However, proportions of PAS-positive cells do not effectively discriminate between forms of abnormal prostatic growth

---

I am indebted to Professor *Helge Stalsberg*, M.D Institute of Medical Biology, University of Tromso Norway for helpful advice and for reading the manuscript Actuary *Ingar Holme*, Cand real, The Norwegian Computing Center Oslo, Norway performed the computer analysis and also gave valuable advice The skilful technical assistance of Mrs. *Vivi Jareg* is greatfully acknowledged

The investigation was supported by grants from *Grosserer N A Stangs Legat for Kreftsykdommers Bekjempelse* and *fru dr Ludvig Larsens legat*

REFERENCES

1 *Abercrombie, M* Estimation of nuclear population from microtome sections. Anat Rec *94* 239 247 1946

2 *Asano M* Basic experimental studies of the pituitary prolactin prostate inter relationships J Urol *93* 87 93, 1965

3 *Bain J* & *E rin, C* Immunofluorescent localization of the LH cell of the human adenohypophysis J clin Endocr *30* 181–184, 1970

4 *Christiansen, P* Urinary follicle stimulating normal

5

servations in carcinoma of the prostate Cancer

6

acute and chronic illness J clin Endocr 917 936 1958

7 *E-rin, C* & *Murray, Shirley* The cells of the human adenohypophysis in pregnancy, thyroid disease

Benoit

l adenohy

de la Recherche Scientifique Paris 1963 p 183–199

8 *Floderus, S* Untersuchungen über den Bau der menschlichen Hypophyse mit besonderer Berucksichtigung der quantitativen mikromorphologischen Verhaltnisse Acta path microbiol scand (Suppl) *53* 1–276, 1944

9 *Geller, J, Baron, Aurora & Kleinman, S* Pituitary luteinizing hormone reserve in elderly men with prostatic disease J Endocr *48* 289–290, 1970

10 *Golden, A* The distribution of cells in the human adenohypophysis Lab Invest *8* 925–938, 1959

11 *Halmi, N S & McCormick, W F* Duration of disease and the delta cell of human hypophysis J clin Endocr *29* 127–129, 1969

12 *Harbitz, T B & Haugen, O A* Histology of the prostate in elderly men A study in an autopsy series Acta path microbiol scand Sect A *80* 756–768, 1972

13 *Haugen, O A & Harbitz T B* Prostatic weight in elderly men An analysis in an autopsy series Acta path microbiol scand Sect A *80* 769 777, 1972

14 *Haugen, O A* Pituitary weight and the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Sect A *81* 204 212, 1973

15 *Heilant, M & Pasteels, J L* Histophysiology of human anterior pituitary In Bajusz, E & Jasmin, G (Eds) Methods and achievements in experimental pathology Vol *3*, pp 250–305 S Karger, Basel New York 1967

16 *Huggins, C & Hodges, C V* Studies on prostatic cancer I The effect of castration of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate Cancer Res *1* 293–297, 1941

17 *Johnsen, S G* A clinical routine method for the quantitative determination of gonadotrophins in 24 hour urine samples. II Normal values for men and women at all age groups from pre puberty to senescence Acta endocr (Kbh) *31* 207 227, 1959

18 *Jones H M* Possible anatomic relations between pituitary body and prostate gland I Normal pituitary body J Urol *41* 84–94 1939

19 *Koppel, M, Metanzie D R & Shimkin, M B* Characteristics of patients with prostatic carcinoma A control case study on 83 autopsy pairs J Urol *98* 229–233, 1967

20 *London, J & Greenwood, F C* Stress and growth hormone release In Gual, C & Ebling F J G (Eds.) Progress in Endocrinology Excerpta Medica Foundation Amsterdam, 1968 p 595–600

21 *Meites, J* Hypothalamic control of prolactin secretion In Wolstenholme G E W & Knight, Julie (Eds) Lactogenic hormones Churchill Livingstone, Edinburgh and London, 1972 p 325–338

22 *Mellgren, J* The anterior pituitary in hyperfunction of the adrenal cortex An anatomical study with special reference to syndroma Morgagni and notes on prostatic hypertrophy Acta path microbiol scand (Suppl) *60* 1–177, 1945

23 *Moore, R A* Benign hypertrophy and carcinoma of the prostate Occurrence and experimental production in animals. In Twombly, G H & Pack, G T (Eds) Endocrinology of neoplastic diseases A symposium by eighteen authors. Oxford University Press, New York, 1947 p 194–212

24 *Nissen Meyer, R* Gonadotropinene og samspillet hypothalamus hypofyse gonader (II) Før puberteten, etter menopausen og hos eldre menn T norske Lægeforen *92* 2177–2180, 1972

25 *Ofner, P* Effects and metabolism of hormones in normal and neoplastic prostate tissue. Vitam and Horm (N Y) *26* 237 291, 1968

26 *Pasteels, J L, Gausset, P, Danguy, A, Ectors, F, Nicoll, C S & Varavudhi, P* Morphology of the lactotropes and somatotropes of man and rhesus monkeys J clin Endocr *34* 959–967, 1972

27 *Pearse, A G E* Differential stain for the human and animal anterior hypophysis Stain Technol *25* 95–102, 1950

28 *Pearse, A G E* Cytological and cytochemical investigations on the foetal and adult hypophysis in various physiological and pathological states J Path Bact *65* 355–370, 1953

29 *Purves, H D* Morphology of the hypophysis related to its function In Young, W C & Corner, G W (Eds) Sex and internal secretions Vol I The Williams & Wilkins Company, Baltimore, 1961 p 161–239

30 *Purves, H D* Cytology of the adenohypophysis In Harris, G W & Donovan, B T (Eds) The pituitary glands Vol 1 Butterworths, London 1966 p 147–232

31 *Rasmussen A T & Herrick, Ruth* A method for the volumetric study of the human hypophysis cerebri with illustrative results Proc Soc exp Biol (N Y) *19* 416–423, 1922

32 *Rasmussen A T* The percentage of the different types of cells in the male adult human hypophysis Amer J Path *5* 263–274, 1929

33

state Cancer *11* 817–828, 1958

34 *Russfield, Agnes B* Combined bioassay and histological study of 73 human hypophysis Cancer *13* 790–803, 1960

35 *Russfield, Agnes B* & *Sommers, S C* Malnutrition and tropic hormone storage in hypophysis Arch Path *75* 564–571, 1963
36 *Ryan, R J* The luteinizing hormone content of human pituitaries I Variations with sex and age J clin Endocr *22* 300–303, 1962
37 *Schalch, D S*, *Parlow, A F*, *Boon, R C* & *Reichlin, S* Measurement of human luteinizing hormone in plasma by radioimmunoassay J clin Invest *47* 665 678, 1968
38 *Scheffe, H* The analysis of variance John Wiley & Sons, Inc, New York 1959 p 25 51
39 *Shanklin, W M* The human hypophysis in cases of burns Acta endocr (Kbh) *21* 1–7, 1956
40 *Snedecor, G W* & *Cochran, W G* Statistical methods. 6th ed The Iowa State University Press, Ames, Iowa 1967 p 114–116
41 *Sommers, S C* Endocrine changes with prostatic carcinoma. Cancer *10* 345–358, 1957
42 *Sommers, S C* Pituitary cell relations to body states Lab Invest *8* 588–621, 1958
43 *Swanson, Heidi* & *Ezrin, C* The natural history of the delta cell of the human adenohypophysis in childhood, adulthood and pregnancy J clin Endocr *20* 952 966 1960
44 *Vermeulen, A*, *Rubens, R* & *Verdonck, L* Testosterone secretion and metabolism in male senescence J clin Endocr *34* 730–735, 1972.
45 *Zubiran, S* & *Gomez Mont, F* Endocrine disturbancies in chronic human malnutrition. Vitam and Horm (N Y) *11* 97 132, 1953

Acta path microbiol scand Section A 81, 425-434 1973

PITUITARY ADENOMAS AND THE HISTOLOGY OF THE PROSTATE IN ELDERLY MEN

An Analysis in an Autopsy Series

OLAV A HAUGEN

Ulleval Hospital, Department of Pathology, University of Oslo, Oslo, Norway

The frequency of pituitary adenomas was recorded in a consecutive autopsy series of men more than 40 years of age Among 170 individuals, not previously subjected to prostatic surgery or treatment with oestrogenic hormones, pituitary adenomas were observed in 33 patients (19 4 per cent) The adenomas were mostly small occupying less than 5 per cent of the section area of the adenohypophysis, and were preferably either chromophobe or mixed acidophil/chromophobe Using multiple regression analysis, the presence of pituitary adenoma was analysed in relation to the histology of the prostate and several other factors Pituitary adenomas occurred more frequently in relation to prostatic carcinoma than to other histological groups, and did not show a significant relationship to other factors tested

Hormonal stimulation apparently initiates, maintains and controls the form and function of the prostatic epitelium (*Scott* 1953) Profound atrophy of the prostate occurs in castrates (*Moore* 1947) and in the absence of pituitary gonadotropins (*Grayhack* 1963) The concept that the prostate is under the dual control of the testis and the adenohypophysis is now generally accepted

In previous reports (*Haugen* 1973 a, 1973 b) evidence was presented which suggested that the pituitary weight and the proportion of PAS-positive cells in the adenohypophysis were higher in men with benign hyperplasia or carcinoma of the prostate than in men with a histologically normal prostate

Small pituitary adenomas have been reported to occur more frequently than expected in relation to benign prostatic hypertrophy (*Close* 1934, *Jones* 1939) or prostatic carcinoma (*Sommers* 1957) However, other investigators were unable to confirm these observations (*Moore* 1947, *Dekker* & *Russfield* 1963, *Koppel et al* 1967)

As part of an investigation of the relationship between pathological growth of the prostate and the morphology of the testis, the adrenal gland and the pituitary gland, the present report describes the findings of pituitary adenomas in a consecutive autopsy series The occurrence of pituitary adenomas has been analysed in relation to prostatic histology and several other factors by single variable analysis and by multiple regression analysis

MATERIAL AND METHODS

The pituitary glands and the prostates from 206 consecutive autopsies of men over 40 years of age were collected during a 3 month period 1967 1968 Thirty-six patients were excluded from the analysis for the following reasons previous prostatic sur

Received 5 ii 73 Accepted 5 ii 73

Requests for reprints should be addressed to Department of Pathology, Ulleval Hospital, Oslo 1

gery (24 patients), oestrogen treatment for clinically manifest prostatic carcinoma (6 patients), secondary tumour invasion of the prostate or testes (4 patients), seminoma of the testis (1 patient), and in one instance the pituitary gland was unsuitable for histological examination due to extensive necrosis. Thus, the analysis was finally based on 170 patients none of whom showed clinical evidence of prostatic carcinoma Apart from seven patients with clinically manifest diabetes mellitus none were known to suffer from other endocrine disorders

The techniques of fixation, microscopical examination of total transverse sections, and histological classification of the prostate have been described previously (*Harbitz & Haugen* 1972) The presence of normal histology (N), benign nodular hyperplasia (BNH), carcinoma (C), atypical glandular proliferation (AGP) or diffuse atrophy (DA) was noted for each gland The occurrence of atypical glandular proliferation was not specified in glands where carcinoma was diagnosed

The pituitary gland was removed, fixed in Helly's fluid and cut in the horizontal plane as previously described (*Haugen* 1973 a, 1973 b) From each gland two central sections, stained according to the PAS Orange G method (*Pearse* 1950), were screened for the presence of adenomas Histological examination was performed without any knowledge of clinical data.

Definition of Pituitary Adenoma

In the present study, pituitary adenoma was defined as groups of chromophil or chromophobe cells which formed distinct nodules clearly contrasting the normal pattern of the adenohypophysis (Fig 1) The presence of a definite capsule surrounding such lesions was not required Reflecting the predominant cell type, the adenomas were classified as acidophil (Orange G positive), basophil (PAS-positive), chromophobe and mixed The sections were screened on three different occasions, and only structures which on all three occasions were interpreted as being adenomas have been included in the analysis

Sections showing adenomas were magnified in a projector and the outer aspects of the pituitary gland, the border between the *pars distalis* and the *pars nervosa* together with the outlines of the adenomas were drawn on paper The size (in arbitrary units) of the adenomas and of the adenohypophysis was measured by planimetry

Statistical Methods

Differences between arithmetic means were tested by a modified Student's t test accounting for unequal variances and numbers of individuals (*Snedecor & Cochran* 1967), and differences between proportions were tested by an x^2 test with correction for continuity (*Armitage* 1971) p values below 0 05 were regarded as statistically significant

Full multiple regression analysis was performed using pituitary adenoma as the *dependent* variable (X_1) In the presence of an adenoma X_1 was given the value 1, otherwise the value 0 The following factors, either bivariate (1,0, labelled X_2 to X_1) or continuous (labelled X_{13} to X_{15}) were treated as *explanatory* (independent) variables

Histology of the Prostate

- X_2 Benign nodular hyperplasia (BNH)
- X_3 Atypical glandular proliferation (AGP)
- X_4 Carcinoma (C)
- X_5 *Diffuse atrophy (DA)*

Cause of Death

- X_6 Cardiovascular disease*
- X_7 Malignant tumour

Duration of Final Illness

- X_8 1 7 days
- X_9 $>$ 7 days

Other

- X_{10} *Steroid hormone treatment***
- X_{11} Diabetes mellitus
- X_{12} Liver cirrhosis
- X *Age*

tiple regression analysis

The analysis was based on a standard program for multiple regression analysis (NRSR) developed at The Norwegian Computing Center, Oslo, and was conducted on a Univac 1108 computer

* includes death from myocardial infarction (48 cases), cerebrovascular and peripheral vascular disease (15 + 7 cases), rheumatic valvular disease (4 cases), miscellaneous cardiovascular disorders (11 cases)

** includes treatment with corticosteroids (7 cases), anabolic steroids (nortestosterone) (3 cases) or both (11 cases)

Fig 1 Circumscribed lesions of the adenohypophysis accepted as small adenomas (A and B) × 30 C and D showing details of other adenomas with a sinusoidal and papillary pattern, respectively × 75 All sections stained with *trichrome* PAS-Orange G

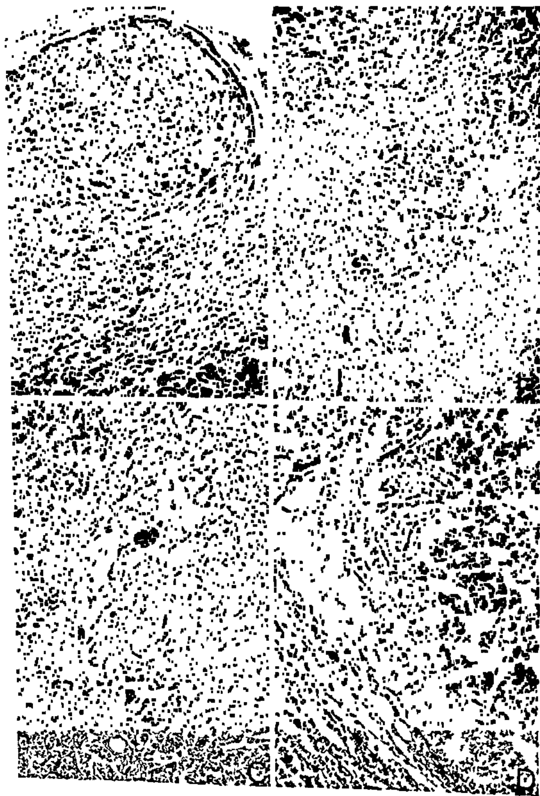

C
D

TABLE 1 *Histological Diagnoses *of the Prostate by Age in 170 Patients*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	1	1	2	0	0	0	0
50–59	10	5	10	3	1	5	0
60–69	7	1	22	15	5	6	1
70–79	0	1	28	21	0	5	1
80 +	0	0	8	9	0	2	0
Total	18	8	70	48	6	18	2

* N = normal histology, DA = diffuse atrophy, BNH = benign nodular hyperplasia, C = carcinoma AGP = atypical glandular proliferation

RESULTS

The histological findings in the prostates of the 170 patients included in the analysis are presented in Table 1

Pituitary adenomas were found in 33 patients (19 4 per cent), three patients showing two adenomas each With one exception these adenomas were small, and mostly occupied less than 5 per cent of the total section area They appeared to be slightly more frequent in the lateral parts of the adenohypophysis and were preferably located near the capsule (Figs 2 and 3) Since the tinctorial properties of the two adenomas occurring within the same hypophysis were identical, the size of these adenomas were combined and in the analysis only counted for 1 adenoma, thus giving a total of 33 adenomas in 33 patients Two adenomas were composed purely of Orange G-positive cells, in five adenomas PAS positive cells dominated, and in 15 adenomas the predominant cell type appeared to be chromophobe In the remaining 11 adenomas the lesions showed a mixture of chromophobe and Orange G positive cells, with occasional PAS positive cells in one adenoma

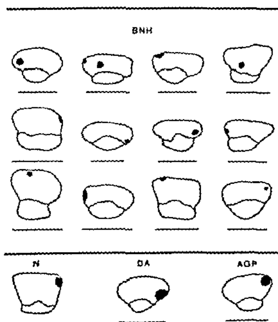


Fig 2 Location and size of pituitary adenomas in patients with benign nodular hyperplasia (BNH), normal histology (N), diffuse atrophy (DA) and atypical glandular proliferation (AGP) of the prostate Scale is indicated by line (natural size = 2 cm.) under each section

Table 2 presents the recorded frequency of pituitary adenomas in relation to age After the age of 80 years the adenomas occurred slightly more frequently than in the younger age groups However the association with age was not particularly strong, and the correlation coefficient was statistically not significant ($p > 0\ 10$) (Table 7) There was a slight decrease of the mean size of the adenomas with age, but the standard deviations were high at all age levels (Table 3), and the correlation coefficient ($r = -0\ 22$) was not statistically significant ($p > 0\ 10$)

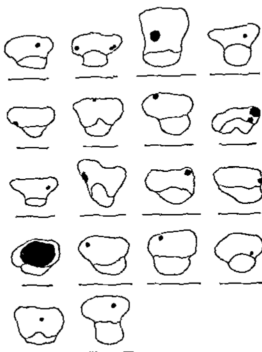


Fig 3 Location and size of pituitary adenomas in patients with carcinoma of the prostate (C) Scale is indicated by line (natural size – 2 cm) under each section

TABLE 2 *Recorded Frequency of Pituitary Adenomas in Relation to Age*

Age	No patients	No with adenomas	Per cent
40–49	4	0	–
50–59	34	5	14 7
60–69	57	11	19 3
70–79	56	11	19 6
80 +	19	6	31 5
All	170	33	19 4

The occurrence of pituitary adenomas in relation to the histology of the prostate appears from Table 4 Patients with carcinoma of the prostate (with or without BNH) showed the highest frequency of pituitary adenomas. The proportion of patients in this group with pituitary adenomas was significantly higher than the proportion of patients

TABLE 3 *Size of Pituitary Adenomas (in arbitrary Units) in Relation to Age**

Age	No of patients with adenomas	Pituitary adenomas Mean size	S D
50–59	5	5 48	4 5
60–69	11	6 33	5 4
70–79	11	4 12	3 9
80 +	5	3 26	3 9
All	32	4 96	4 5

S D *Standard deviation*
* One large adenoma (size in arbitrary units 169 6) excluded

with pituitary adenomas in the rest of the material ($X^2 = 8.30$, $p < 0.005$) If patients with BNH alone and patients with C+BNH were compared, the frequency of pituitary adenomas was seen to be significantly higher among the latter ($X^2 = 4.19$, $p < 0.05$)

Pure acidophil adenomas were encountered in two patients who both had prostatic carcinoma (Table 5) The majority of adenomas occurring in patients with C of the prostate was, however, chromophobe Amongst 12 adenomas encountered in the pituitary of patients with BNH alone, six were mixed chromophobe/Orange G positive Adenomas which were composed purely from PAS positive cells were observed in five instances in patients with either C+BNH or BNH of the prostate The size (in arbitrary units) of the adenomas found in patients with BNH and C was on an average rather close to each other (Table 6)

Multiple Regression Analysis

In the course of the multiple regression analysis simple correlation analysis was also performed, the correlation coefficients are presented in Table 7 Among the histological diagnoses both BNH and C showed a positive relationship to the presence of pituitary adenomas the latter alone being highly significant The relationship to other variables were negligible and not statistically significant

TABLE 4 *Recorded Frequency of Pituitary Adenomas in Relation to the Histology of the Prostate**

Histology of the prostate	No of patients	No of patients with adenomas	Per cent
N	18	1	5 5
DA	8	1	12 5
BNH	70	12	17 5
C + BNH	48	17	35 4
C	6	1	16 6
AGP + BNH	18	1	5 5
AGP	2	0	–
All	170	33	19 4

* *For abbreviations, see Table 1*

TABLE 5 *Classification of Pituitary Adenomas in Relation to the Histology of the Prostate**

Histology of the prostate	Total no of adenomas	No of acidophil	No of basophil	No of chromophobe	No of mixed
N	1	–	–	–	1
DA	1	–		1	–
BNH	12	–	1	5	6
C and C + BNH	18	2	4	9	3
AGP and AGP + BNH	1	–	–	–	1
All	33	2	5	15	11

* For abbreviations see Table 1

TABLE 6 *Mean Size (in arbitrary Units and in per cent of total Section Area of the Adenohypophysis) of Pituitary Adenomas in Relation to Benign Hyperplasia (BNH) and Carcinoma of the Prostate (C and C + BNH)*

Histology of the prostate	No of patients	Mean size	S D	Per cent of adenohypophysis
BNH	12	4 13	3 1	3 40
C and C + BNH*	17	4 72	5 1	4 12

* One large adenoma (size in arbitrary units 169 9 involving 61 1 per cent of the adenohypophysis) excluded
S D Standard deviation

The total set of variables was included in the full regression analysis (Table 8) C of the prostate still showed a positive and statistically significant relationship to the presence of pituitary adenoma, and BNH was selected second to C The regression coefficient for BNH however was not statistically significant

TABLE 7 *Relationship between the Presence of Pituitary Adenomas and Variables listed X_2 to X_{15} Simple Correlation Analysis*

Explanatory variables	Y_1 Pituitary adenoma (n_1 = 33) Correlation coefficient	Significant at level
*Histology of the prostate**		
X_2 BNH (n = 136)	0,140	0 070
X_3 AGP (n = 20)	–0 133	0 084
X_4 C (n = 54)	0,240	0 002
X_5 DA (n – 8)	–0 038	0,615
Cause of death		
X_6 Cardiovascular disease (n 85)	–0,104	0,177
X_7 Malignant tumour (n – 44)	0 083	0,279
Duration of final illness		
X_8 1 7 days (n – 35)	0 007	0,922
X_9 >7 days (n = 99)	0,023	0 760
Other		
X_{10} Steroid hormone treatment (n = 23)	0 066	0 387
X_{11} Diabetes mellitus (n 7)	–0 027	0,728
X_{12} Liver cirrhosis (n 5)	–0 085	0 268
X_{13} Age (n = 170)	0,117	0 129
X_{14} Body weight (n 170)	–0 079	0 306
X_{15} Body length (n = 170)	0,074	0 335

* For abbreviations, see Table 1 n_1 Number of cases with pituitary adenomas
n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continuous variables)

COMMENT

The question whether the lesions of the adenohypophysis which in the present study have been termed adenomas, in fact represent true neoplasms in their early stage of evolution or foci of atypical hyperplasia is not merely a semantic one *Willis* (1967) states that the pituitary adenomas in their early stages are often not encapsulated, and probably commence as focal hyperplasia which go on to tumour formation, whilst *Evans* (1968) believes that this is true only occasionally However, both authors agree that it is difficult to distinguish between a small adenoma and a focus of atypical hyperplasia

In large autopsy series microscopic anterior lobe adenomas have been reported to be common (*Close* 1934, *Costello* 1936, *Sommers* 1958 *McCormick* & *Halmi* 1971), the frequencies varying from 6 5 to 22 5 per cent Obviously, the great variation may in part be attributed to differences in criteria, and secondly may also be influenced by the levels and number of sections studied From the present data it may be assumed that several adenomas would have escaped detection if sections had been cut in the mid sagittal plane of the gland However since only two sections were examined in the present series, it is also likely that small adenomas in other parts of the hypophysis may have escaped detection

Significant progress has been made in the correlation of cell type and specific hormone production of the adenohypophysis in man by application of histochemical and immunochemical methods (*Herlant* & *Pasteels* 1967) Yet, since no single staining method is available which specifically stains the different types of pituitary cells according to their hormonal activity, it is still customary and convenient to classify pituitary adenomas as acidophil (Orange G positive), basophil (PAS-

TABLE 8 *Pituitary Adenomas in Relation to Variables listed X_2 to X_{15} Full Regression Analysis*

Explanatory variables*	X_1 Pituitary adenoma (n_1=33) Partial correlation coefficient	Partial regression coefficient	Significant at level
X_4 C (n = 54)	0 163	14 81	0 041
X_2 BNH (n = 136)	0 105	12 33	0 189
X_{15} Body length (n = 170)	0 082	0 005	0 305
X_3 AGP (n = 20)	-0 080	-0 009	0 318
X_6 Cardiovascular disease (n = 85)	-0 068	-0 006	0 396
X_{14} Body weight (n = 170)	-0 062	-0 002	0 437
X_{12} Liver cirrhosis (n = 5)	-0 061	-0 014	0 446
X_9 >7 days (n = 99)	-0 051	-0 005	0 525
X_{13} Age (n = 170)	0 047	0 002	0 554
X_{10} Steroid hormone treatment (n = 23)	0 041	0 005	0 606
X_8 1 7 days (n = 35)	0 038	0 004	0 631
X_{11} Diabetes mellitus (n = 7)	-0 030	-0 006	0 700
X_5 DA (n = 8)	0 020	0 004	0 799
X_7 Malignant tumour (n = 44)	0 014	0 001	0 856

Multiple correlation coefficient (R) 0 326
* For abbreviations of histological diagnoses, see Table 1
n_1 and n For explanation, see Table 7

positive) and chromophobe (*Kernohan* & *Sayre* 1956, *Willis* 1967, *Evans* 1968, *Currie* 1971) However, this classification has been strongly criticized by a number of workers (*Pearse* 1962, *Russfield* 1968, *McCormick* & *Halmi* 1971) since the existence of chromophobe cells as a separate functional cell type is currently in doubt

Previous studies of the frequency of pituitary adenomas in relation to abnormal growth of the prostate have produced conflicting results *Close* (1934) examined a large number of pituitary sections from 10 patients with adenoma of the prostate among whom eight had pituitary adenomas, against five among 50 controls *Jones* (1939) examined a large number of patients and found prostatic enlargement to be more common in patients with subclinical pituitary adenomas than among age matched controls However, since histological examination of the prostate was not performed, the significance of this observation is obscure Among 32 patients with prostatic carcinoma, *Sommers* (1957) found six with pituitary adenomas against seven adenomas in 128 controls His materials were collected from autopsy files, and it is likely that only a small portion of the prostate had been histologically examined, rendering the value of his controls rather doubtful Objections may also be raised against other investigations (*Moore* 1947, *Dekker* & *Russfield* 1963, *Koppel et al* 1967) in which no association between the presense of pituitary adenomas with benign prostatic hyperplasia or carcinoma could be demonstrated This criticism would include both the small number of patients examined and doubtful controls

The majority of adenomas encountered in the pituitary gland in the present series were small and dominated by either chromophobe cells or showed a mixture of chromophobe and Orange G positive cells The poor correspondence between tinctorial properties and hormonal activity of the pituitary cells gives an inadequate basis for a detailed discussion of the possible functional significance of these lesions It is possible, however, that adenomas showing dominance of acidophil cells or being mixed acidophil/chromophobe could contain, store or produce growth hormone or prolactin The majority of Orange

G positive cells in man contain the growth hormone antigen (*Beck et al* 1966, *Haugen & Beck* 1969) while a minor proportion stains specifically for prolactin (*Pasteels et al* 1972) Recent studies have given evidence of a secretory activity in chromophobe cells (for references, see *Russfeld* 1968), and electron microscopic studies of chromophobe adenomas have uncovered the presence of secretory granules (*Schelin* 1962) *McCormick & Halmi* (1971) reported that the majority (about 60 per cent) of the adenomas with "agranular cells' were acidophil, nearly 20 per cent derived from the mucoid cells and a similar proportion was of mixed cell origin Thus, the chromophobe cells appear to be a mixture of different cell types, and their hormonal activity may presumably be equally variable

In the present investigation pituitary adenomas were significantly more frequent among patients with prostatic carcinoma than among patients without prostatic malignancy Taking several factors into account as possible explanatory variables in the multiple regression analysis, carcinoma of the prostate still showed a significant relationship to the presence of pituitary adenoma, while the relationship to BNH was not statistically significant This observation does not necessarily imply a causal relationship between pituitary adenomas and abnormal growth of the prostate These conditions could all be related to a common, yet undetermined factor, leading to formation of tumours in the pituitary gland and the prostate Experimental studies (*Clifton 1959, Furth 1969*) and limited observations in man (for references, see *Russfield* 1967) suggest that deficiency of pituitary target organs may be involved in the pathogenesis of pituitary tumours

This subject will be further discussed in a forthcoming paper on the morphological characters of the testes the pituitary gland and the adrenal glands in relation to the histology of the prostate in elderly men comprised in the series reported here

I am indebted to Professors *Helge Stalsberg*, M.D and *Knut Westlund*, M D, Institute of Medical Biology, University of Tromso, Norway for helpful advice in matters concerning the statistical analysis and for reading the manuscript Actuary *Ingar Holme*, Cand real, The Norwegian Computing Center, Oslo, Norway performed the computer analysis and gave valuable advice

This study was financially supported by grants from *Grosserer V A Stangs Legat for Kreftsykdommers Bekjempelse* and *Dr Alexander Malthes Legat*

REFERENCES

1 *Armitage, P* Statistical methods in medical research Blackwell Scientific Publications Oxford and Edinburgh 1971 p 135

2 *Beck J S, Ellis Susan T, Legge, J S, Porteous, I B, Currie, A R & Read, C H* Characterisation of an antiserum to human growth hormone and the localisation of the hormone in the normal adult adenohypophysis J Path Bact *91* 531–538 1966

3 *Clifton K H* Problems in experimental tumorigenesis of the pituitary gland, gonads adrenal cortices and mammary glands a review Cancer Res *19* 2 22, 1959

4 *Close, H G* The incidence of adenoma of the pituitary body in some types of new growth Lancet *I* 732 734 1934

5 *Costello, R T* Subclinical adenoma of the pituitary gland Amer J Path *12* 205–216, 1936

6 *Currie, A R* Pituitary gland In Anderson, W A D (Ed) Pathology 6th ed The C V Mosby Company, St Louis 1971 p 1403–1430

7 *Dekker, A & Russfield Agnes B* Pituitary tropic hormone studies and morphological observations in carcinoma of the prostate Cancer *16* 743–750, 1963

8 *Evans R W* Histological appearances of tumours With a consideration of their histogenesis and certain aspects of their clinical features and behaviour 2nd ed E & S Livingstone Ltd, Edinburgh & London 1968 p 457–472

9 *Furth, J* Pituitary cybernetics and neoplasia Harvey Lect 63 47–71, 1969

10 *Grayhack J T* Pituitary factors influencing growth of the prostate In Vollmer, F P (Ed) Biology of the prostate and related tissues Nat Cancer Inst Monogr *12* 189–199, 1963

11 *Harbitz T B & Haugen, O A* Histology of the prostate in elderly men A study in an autopsy series. Acta path microbiol scand. Section A *80* 756–768, 1972

12 *Haugen, O A & Beck, J S* Immunofluorescence studies with antisera to human growth

hormone and human placental lactogen on adenohypophysis of pregnant and parturient women J Path *98* 97–104, 1969

13 *Haugen, O A* Pituitary weight and the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *81* 204–212, 1973a

14 *Haugen, O A* Distribution of pituitary cell types in relation to the histology of the prostate in elderly men An analysis in an autopsy series. Acta path microbiol scand Section A *81* 411–424, 1973b

15 *Herlant, M* & *Pasteels, J L* Histophysiology of human anterior pituitary In Bajusz, E & Jasmin, G (Eds) Meth Achiev exp Path Vol 3 S Karger, Basel and New York 1967 p 250–305

16 *Jones, H M* Possible anatomic relations between the pituitary body and the prostate gland II The pathological pituitary body J Urol *42* 50–56, 1939

17 *Kernohan, J W* & *Sayre, G P* Tumors of the pituitary gland and infundibulum Atlas of tumor pathology Section X- Fascicle 36 Armed Forces Institute of Pathology, Washington, D C 1956 81 pp

18 *Koppel, M , Meranzie, D R* & *Shimkin, M B* Characteristics of patients with prostatic carcinoma A control case study on 83 autopsy pairs J Urol *98* 229 233, 1967

19 *McCormick, W F* & *Halmi, N S* Absence of chromophobe adenomas from a large series of pituitary tumors Arch Path *92* 231–238, 1971

20 *Moore, R A* Benign hypertrophy and carcinoma of the prostate Occurrence and experimental production in animals In Twombly, G H & Pack, G T (Eds) Endocrinology of neoplastic diseases. A symposium by eighteen authors Oxford University press, New York 1947 p 194–212

21 *Pasteels, J L , Gausset, P , Danguy, A , Ectors, F , Nicoll, C S* & *Varavudhi, P* Morphology of the lactotropes and somatotropes of man and rhesus monkeys J clin Endocr *34* 959–967, 1972

22 *Pearse, A G E* Differential stain for the human and animal anterior hypophysis Stain Technol *25* 95–102, 1950

23 *Pearse, A G E* Cytology and cytochemistry of adenomas of the human hypophysis Acta Un int Cancr *18* 302–304, 1962

24 *Russfield, Agnes B* Pituitary tumors In Sommers, S C (Ed) Pathology Annual Butterworths, London 1967 p 314–350

25 *Russfield, Agnes B* Adenohypophysis. In Bloodworth, J M B jr (Ed) Endocrine Pathology Williams & Wilkins Co, Baltimore 1968 p 75–116

26 *Schelin, U* Chromophobe and acidophil adenomas of the human pituitary gland A light and electron microscopic study Acta path. microbiol scand (Suppl) *158* 80 pp 1962

27 *Scott, W W* What makes the prostate grow? J Urol *70* 477–488, 1953

28 *Snedecor, G W* & *Cochran, W G* Statistical methods 6th ed The Iowa State University Press, Ames Iowa 1967 p 114–116

29 *Sommers, S C* Endocrine changes *with prostatic* carcinoma. Cancer *10* 345–358, 1957

30 *Sommers, S C* Pituitary cell relations to body states Lab Invest *8* 588–621, 1958

31 *Willis, R A* Pathology of tumours 4th. ed. Butterworths, London 1967 p 640–645

Acta path microbiol scand Section A 81, 435–444, 1973

LOSS OF EPITHELIAL BLOOD GROUP SUBSTANCE A IN ORAL CARCINOMAS

E Dabelsteen & J J Pindborg

Department of Oral Pathology, Royal Dental College,
Dental Department, University Hospital, Copenhagen Denmark

Tissues from 12 oral squamous cell carcinomas were investigated for the presence of blood group antigen A The antigen reactivity in the carcinomas was compared, by titration, to the reactivity of adjacent normal epithelium included in the same specimen Normal epithelium always reacted positively In 6 of 12 specimens the malignant cells gave a completely negative reaction In 5 specimens there was a patchy distribution of positive reaction on cells within the carcinoma However, these cells possessed less antigen than the normal epithelial cells at the edges of the surgical specimens In one specimen the majority of cells in the carcinoma showed no decrease in reactivity In three cases exhibiting slight epithelial atypia adjacent to the tumour, the reactivity of blood group substances in these areas was found to be higher than in the carcinomas but lower than in the normal mucosa

There is considerable evidence that malignant development involves important changes of the cell surface (23) These changes may include the appearance of new antigens (15, 18, 25, 29) or the loss of antigens normally present (2, 10, 24) Partial or complete loss of blood group antigens A and B has been reported for both premalignant and malignant lesions developing from epithelium in which such substances are normally present, e g in oral mucosa (8), in cervical epithelium (10, 12), gastrointestinal mucosa (11, 16, 28) and pancreas (13)

In normal oral mucosa the blood group antigens A and B have been demonstrated on the cell membranes of the epithelial cells (1, 5 19, 30) *Kovarik et al* (21) showed, by means of the mixed cell agglutination technique, that two out of 5 carcinomas from the tongue reacted positively for iso-antigens A or B, the other three cases investigated were negative Later, *Prendergast* (26), studying the same antigens in 8 carcinomas of the oral mucosa by means of an immunofluorescence staining method, confirmed *Kovarik's* results A recent publication (5) states that the amount of antigen in the normal oral mucosa varies from person to person and that sometimes these in the non-secretor group (17) have so little antigen that it can hardly be detected Therefore, any description of blood group antigens in oral carcinomas must be based on a comparison with the amount of antigen in normal mucosa, this has not been the case in previously published work

The present report describes the distribution of blood group antigens in oral squamous cell carcinomas on the basis of quantitative comparison with adjacent normal mucosa

Received 13 i 73 Accepted 24 i 73

Requests for reprints should be addressed to *E Dabelsteen*, Department of Oral Pathology, Royal Dental College, 4 Universitetsparken DK 2100 Copenhagen Ø Denmark.

MATERIALS AND METHODS

The material comprised surgical specimens from 12 squamous cell carcinomas of the oral cavity The

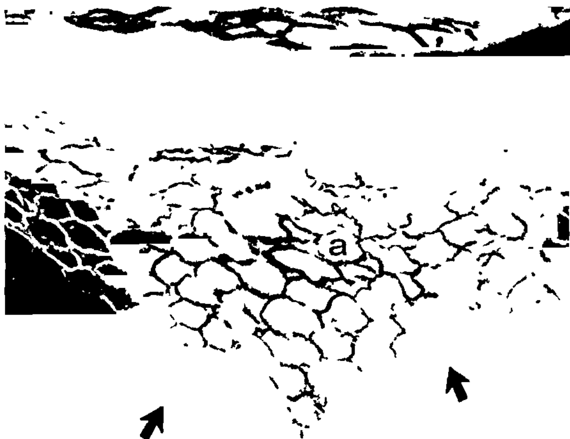


Fig 1 Normal buccal mucosa Immunofluorescence stain ng The bright intercellular spaces in the p nous cell layer (a) indicate the presence of blood group antigen A The arrows ind cate tl e basal membrane area × 200

patients (6 men and 6 women) all belonged to blood group A Eleven of the specimens contained histologically normal mucosa adjacent to the carci noma. In one case where no normal mucosa was present in the surgical specimen a biopsy of clini cally normal lower l p mucosa was obtained in addit on

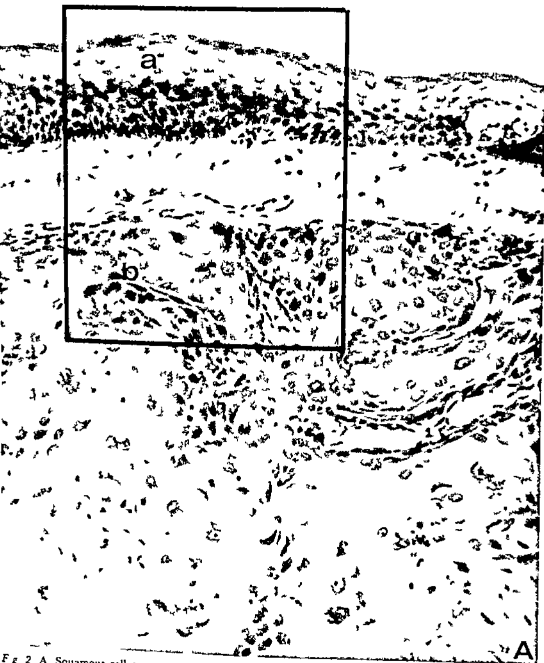
The blood group antigens on the epithelial cells were investigated by a double layer immunofluo rescence technique (9 32) with blood group anti sera and conjugate as described by *Dabelsteen* (5) The fluorescence microscope filter system staining technique and control reactions are identical to those described in previous papers (9 27)

In every case the amount of blood group sub-stances in the tissue was estimated by a two-fold serial titration the reciprocal of the highest dilution which gave a positive reaction was regarded as the endpoint titre The reactions were read as positive or negative If different endpoint titres were obtai ned in different areas of the same section tl s was registered

RESULTS

In all specimens investigated, normal mucosa reacted positively, the cell membranes of tl e spinous layer stained uniformly bright green (*Fig* 1)

In 6 of the 12 specimens the carcinoma gave a completely negative reaction to all concentrations of antibody (Fig 2) In 5 specimens there was a patchy distribution of positive reaction on cells within the carci noma (Fig 4) Titrations in this material all showed that the carcinoma possessed less an



F g 2 A Squamous cell carc noma of the oral mucosa (b) co ered by normal oral mucosa (a) Haematoxyl n and eos n stain ng × 95 B and C h gh power of area nd cated in A (For B and C see next pages)



Fig 1 Normal buccal mucosa Immunofluorescence staining The bright intercellular spaces in the spinous cell layer (a) indicate the presence of blood group antigen A The arrows indicate the basal membrane area × 200

patients (6 men and 6 women) all belonged to blood group A Eleven of the specimens contained histologically normal mucosa adjacent to the carcinoma. In one case where no normal mucosa was present in the surgical specimen a biopsy of clinically normal lower lip mucosa was obtained in addition

The blood group antigens on the epithelial cells were investigated by a double layer immunofluorescence technique (9 32) with blood group antisera and conjugate as described by *Dabelsteen* (5) The fluorescence microscope filter system staining technique and control reactions are identical to those described in previous papers (9 27)

In every case the amount of blood group substances in the tissue was estimated by a two-fold serial titration the reciprocal of the highest dilution which gave a positive reaction was regarded as the endpoint titre. The reactions were read as positive or negative If different endpoint titres were obtained in different areas of the same section this was registered

RESULTS

In all specimens investigated normal mucosa reacted positively, the cell membranes of the spinous layer stained uniformly bright green (Fig 1)

In 6 of the 12 specimens the carcinoma gave a completely negative reaction to all concentrations of antibody (Fig 2) In 5 specimens there was a patchy distribution of positive reaction on cells within the carcinoma (Fig 4) Titrations in this material all showed that the carcinoma possessed less an

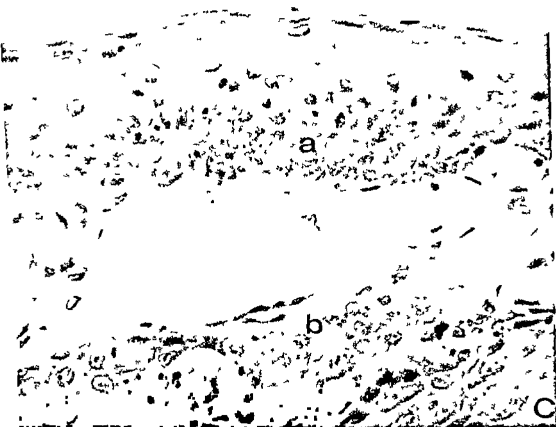


Fig 2 C Haematoxylin and eosin staining of neighbouring section

tigen than the normal epithelium at the edge of the sugical specimen

In one specimen, the majority of cells in the carcinoma reacted positively to endpoint titres of the same magnitude as the normal epithelium distant from the carcinoma (Fig 3) The uninvolved epithelium at the edge of the carcinoma had, on the other hand, an endpoint titre lower than the epithelium distant from the carcinoma In three cases the mucosa adjacent to the tumour showed slight epithelial atypia (dysplasia) as judged by the criteria described by Dabelsteen et al (7) In these areas with epithelial atypia the endpoint titre was lower than in the normal epithelium but higher than in the carcinoma (Table 1)

DISCUSSION

The present work has evaluated the amount of blood group antigen A in oral carcinomas from 12 patients by making comparison with the amount of antigen in normal mucosa of the same patient It has shown that there is a marked decrease in quantity of the antigen in most carcinomas Titrations have revealed that in some cases loss of antigen is accompanied by the retention of areas within the carcinoma still reacting positively at the highest concentrations of antibody, however, these areas have endpoint titres much lower than the normal epithelium at the edge of the specimen This difference is only revealed through titration and the value of this method of quantitation of antigen against antibody is thus emphasized In one case it was not possible to demonstrate any decrease of anti



Fig 4 Squamous cell carcinoma of the oral cavity Immunofluorescence staining Area showing patchy distribution of blood group antigen A a Positive reacting cells b negative re acting groups of cells × 450

the glycolipids processing blood groups A and B in normal cell surfaces The loss of A and B blood group activity accompanied by the appearance of Le^a activity in adenocarcinomas, as reported by *Hakomori et al* (16) is in agreement with our findings that the blood group A substance disappears in carcinomas Furthermore, they are in agreement with un published findings performed in this laboratory which seem to indicate the presence of Le^a activity in the carcinomas investigated

This seems to indicate that the blood group antigens disappear in carcinomas because of an aberration in synthesis rather than by being masked under an abnormal cell coat as has been suggested by *Currie* (4)

Gold et al (14) have recently shown that purified human anti-A antibody is able to react with the carcinoembryonic antigen (CEA), which is a tumour specific constituent of the tumour cell surface in adenocarcinomas of the human alimentary tract They have thus illustrated that cross reactivity can exist between blood group A antigen and a tumour antigen This may explain the strong reactivity for blood group antigen A found in one of the cases investigated in the study

In conclusion the present study has shown that blood group substances decrease in amount or disappear in some oral carcinomas Furthermore, it has been shown that they decrease in amount in mucosa exhibiting slight epithelial atypia adjacent to carcinoma This leads to the suggestion that the change in antigen pattern at the cell surfaces is a tumour-associated change which in some

cases may precede the carcinomatous development The study has shown that it is important to quantitate the antigen when comparing normal mucosa and carcinomas

---

The present work was supported by *Daell Fonden, Copenhagen, Denmark* and Grant DE 1358 from *The National Institute of Dental Research, National Institutes of Health, Bethesda Maryland U.S.A.*

REFERENCES

1 *Brandtzaeg, P* Localization of blood group substances A and B in alcohol fixed human gingivae by indirect immunofluorescent technique Acta odont scand *23* 335–345 1965

2 *Burtin P, Sabine, M C & von Kleist, S* Loss of a normal colonic membrane antigen in the human cancers of the colon Cancer Res. *31*

3

epithelial cells at different stages of differentiation and in malignancy In Cell Differentiation, edited by *R Harris P Allin & D Viza*, Munksgaard, Copenhagen 1972

4 *Currie, G A & Bagshawe, K D* The role of sialic acid in antigenic expression Further studies of the Landschutz ascites tumor Brit J Cancer *22* 843–853, 1968

5 *Dabelsteen, E* Quantitative determination of blood group substances A of oral epithelial cells by immunofluorescence and immunoperoxidase methods. Acta path microbiol scand Section A *80* 847–853 1972

6 *Dabelsteen E Fejerskov, O Francois D & Sundstrom B* Ultrastructural localisation of blood group antigen A and cell coat on oral epithelial cells Abstract No 4 Annual Meeting of Scandinavian Division of the International Association for Dental Research. In Scand J dent Res *80* No 6 *1972*

7 *Dabelsteen E Roed Petersen B, Smith C J & Pindborg J J* The limitations of exfoliative cytology for the detection of epithelial atypia in oral leukoplakias Brit J Cancer *25* 21 24 1971

8 *Dabelsteen F & Fulling H J* A preliminary study of blood group substances A and B in oral epithelium exhibiting atypia Scand J dent Res *79* 387–393 1971

9 *Dabelsteen E & Rygaard, J* A sensitive immunofluorescence technique for detecting blood group substances A and B Acta path microbiol scand Section A *80* 433–439, 1972

10 *Davidsohn, J* Early immunologic diagnosis and prognosis of carcinoma Amer J clin Path. *57* 715–730, 1972

11 *Davidsohn, J, Kovarik, S & Lee, C L* A, B and O substances in gastrointestinal carcinoma Arch Path *81* 381–390, 1966

12 *Davidsohn J, Kovarik, S & Ni, L Y* Isoantigens A, B, and H in benign and malignant lesions of the cervix Arch Path *87* 306–314, 1969

13 *Davidsohn, J, Ni, L Y & Stejskal, R* Tissue isoantigens A, B and H in carcinoma of the pancreas *Cancer Res 31 1244–1250 1971*

14 *Gold, J M, Freedman, S O & Gold, P* Human anti CEA antibodies detected by radioimmunoelectro-phoresis *Nature New Biology 239* 60–62, 1972

15 *Gold P & Freedman, S O* Specific carcino-

16

Jeanloz R W Immunologic relationship between blood group substances and a fucose-containing glycolipid of human adenocarcinoma J Immunol *98* 31–38, 1967

17

18

Immune Surveillance edited by R T Smith & Maurice Landy Academic Press New York London 1970, Chap IV, p 263

19 *Holborow E J, Brown P G Glynn, L E, Hawes, M D, Gresham, G A O Brien T F & Coombs, R R A* The distribution of the blood group A antigen in human tissues Brit J exp Path *41* 430–437, 1960

20 *Inbar, M, Ben Bassat, H & Sachs, L* Membrane changes associated with malignancy Nature New Biology *236* 3–4, 1972

21 *Kovarik, S, Davidsohn J & Stejskal, R* ABO antigens in cancer Arch Path *86* 12–21, 1968

22 *Mallucci, L, Poste, G H & Wells, V* Synthesis of cell coat in normal and tranformed cells *Nature New Biology 235* 222–223, 1972

23 *Martinez Palomo, A* The surface coats of animal cells. Intern Rev Cytol. *29* 29–75, 1970

24 *Nairn R C, Richmond, H G, McEntegart, M G & Fothergill, J E* Immunological differences between normal and malignant cells Brit Med J *2* 1335–1340 1960

25 *Prehn, R T & Main, J M* Immunity to methylcholanthrene-induced sarcomas J nat Cancer Inst *18* 769–778, 1957

26 *Prendergast R C, Toto P D & Gargiulo A W* Reactivity of blood group substances of neoplastic oral epithelium J dent Res. *47* 306–310 1968

27 *Rygaard, J & Olsen W* Determination of

characteristics of interference filters Ann N Y Acad Sci *177* 430–433, 1971
28 *Sheahan, D G, Horowitz, S A, Zamcheck, N* Detection of epithelial ABH isoantigens in primary gastric neoplasms and in metastatic cancer Digestive Diseases *16* 961–969, 1971
29 *Sjogren, H O* Transplantation methods as a tool for detection of tumor specific antigens Progr exp Tumor Res *6* 289–322, 1965
30 *Szulman, A E* The histological distribution of blood group substances A and B in man J exp Med *111* 785–800, 1960
31 *Warren, L, Critchley, D & Macpherson, I* Surface glycoproteins and glycolipids of chicken embryo cells transformed by a temperature sensitive mutant of Rous Sarcoma Virus Nature *235* 275–277, 1972
32 *Weller, T H & Coons, A H* Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro Proc. Soc. exp Biol (N Y) *80* 789–794, 1954

Acta path microbiol scand. Section A 81, 445–452, 1973

A TRANSPLANTABLE ASCITES RHABDOMYOSARCOMA IN THE RAT

Michael Petri

The University Institute of Pathological Anatomy,
University of Copenhagen

In a previous reported series of rats, rhabdomyosarcomas developed in the peritoneal cavity after growth and regression of the transplantable Yoshida ascites sarcoma, infected by the microsporidian parasite *Nosema cuniculi*. These tumours have now been reproduced in a new series of rats under the same conditions and converted into a transplanted ascites sarcoma with 99 per cent takes and 99 per cent death rate. Subcutaneous transplantation of the ascites tumour is followed by the formation of a solid tumour. The microscopic structure is described.

In a series of white rats, persistence of the Yoshida ascites sarcoma for an extended period of time was followed by regression of the tumour. This was due to an intracellular infection of the sarcoma cells with the protozoan parasite *Nosema cuniculi*, as rats transplanted with the non infected sarcoma rarely survive (*Petri* 1966). The parasite belongs to the *Microsporida*, a group of parasites with numerous species, known from diseases of lower animals and recently recognized in many vertebrates (see *Petri* 1969). It is the cause of Nosematosis in laboratory rodents, a latent disease which may become activated under experimental conditions. Transplanted ascites tumours in mice and rats in particular may become infected. The infection is able to persist for several years with only insignificant alterations in the behaviour of the tumour and may therefor remain unrecognized (*Petri* 1965, 1966, 1969).

In rats thus surviving the Nosema infected Yoshida sarcoma, 37 per cent developed metastasizing solid, intraperitoneal rhabdomyosarcomas after a latent period of from 5 to 22 months (*Petri* 1968). There was a certain histological similarity between the earliest of these tumours and the solid form of the Yoshida sarcoma, but otherwise the two sarcomas seemed quite distinct.

These late sarcomas have been reproduced in a new series of animals and one of them established as a transplanted ascites tumour.

MATERIALS AND METHODS

The experiments were carried out in another institute*) than those previously described by the author (*Petri* 1968). The rats were kept in plastic cages on a standard regimen ... reciprocal skin graft in the course of 100 days, and therefore seem to comply with generally accepted criteria for inbred strains (*Sparck* 1968).

Received 22.x.72. Accepted 6.i.73.

Requests for reprints should be addressed to: Dr. *Michael Petri*, The University Institute of Pathological Anatomy, Juliane Mariesvej 16, DK 2100 Copenhagen Ø, Denmark.

*) University Institute of Pathological Anatomy.

The transplanted *rhabdomyosarcoma* originated in a female rat 193 days after transplantation of the infected sarcoma, in the fibrous scar tissue *formed* after regression (*Petri* 1968) The tumour was minced with scissors and 0 25 ml injected intraperitoneally *into two rats* A haemorrhagic ascites developed in both and further passages were made by intraperitoneal or subcutaneous injection of 0 5 *ml*

Erythrocytes were counted in a Neubauer counting chamber by standard method for peripheral blood after dilution with Hayem s fluid

Dry smears were fixed by methanol and stained with Giemsa or methyl green pyronine Tissues were fixed in Carnoy s fluid or neutral, buffered formalin, embedded in paraffin, and stained by standard methods

tion by osmium Embedding in Vestopal, staining by lead citrate and uranyl acetate

RESULTS

A Late Sarcomas

At the time of appearance of the last tumour observed, 11 out of 61 survivors (18 per cent) developed rhabdomyosarcomas of the type previously reported (*Petri* 1968) A number of surviving rats which accidentally were exposed to fatal concentrations of ammonia vapours or died from pulmonary sequelae, were excluded from the material The period from transplantation of the *Nosema* infected Yoshida sarcoma until appearance of the late sarcomas ranged from 169 to 472 days the average being 243 days

B Transplanted Sarcomas

In the first two passages, the survival time of 4 rats was 50, 40, 41 and 39 days after intraperitoneal transplantation During the subsequent 11 passages in 49 female and 49 male rats, 2 to 3 months of age the average time was 20 5 and 20 0 days, the maximum and minimum being 29 and 14 days In only one rat no tumour developed

Visible distension of the abdomen was seen 4 to 5 days before death at which time as much as 18 ml of highly sanguinolent ascites was found The tumour invades the omenta and mesentery which are converted into nodular masses and small nodules are regulary found in the spleen and pancreas. While metastases to the lungs and mediastinal lymph nodes were produced during the first 6 passages, no distant metastases could be demonstrated in later passages

By subcutaneous transplantation of 0 5 ml ascites in 10 rats a tumour was *formed in 9*, the survival time ranging from 44 to 56 days, an average of 52 4 days At autopsy the tumours were found to have a large central necrosis There were no distant metastases.

Composition and microscopic structure Erythrocytes greatly outnumbered nucleated cells in the *ascites* tumour (1 84 million per μl, passage no 14) in all passages. Except for the rather few white blood cells, the majority of nucleated cells consists of 2 types A large, polymorphous, intensely basophilic and pyroninophilic cell, regarded as the sarcoma cell, most often in clusters of a few to many, and a cell with abundant, vacuolated cytoplasm and a small nucleus, perhaps desquamated mesothelial cells or fat cells A few of these contain phagocytosed material (Figs. 1, 2) The aggregation was observed also by phase contrast microscopy of fresh tumour ascites A number of the sarcoma cells formed small giant cells (Fig 3)

The *solid* form of the sarcoma is formed either as metastatic nodules in the abdominal cavity or after subcutaneous transplantation of the ascites sarcoma The original tumour was composed of highly differentiated muscle cells with cross striations as well as less differentiated, cellular tissue with smaller, more or less elongated cells arranged in bundles and

---

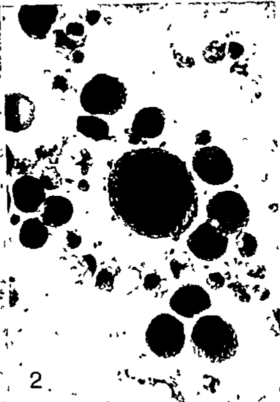
Fig 1 Large cluster of sarcoma cells. The dark staining is due to heavy basophilia Giemsa (63 ×)

Fig 2 Polymorphous sarcoma cells and vacuolated cells The fluid also contains leucocytes and a large number of erythrocytes Giemsa (560 ×)

Fig 3 Same specimen as Fig 2 A small giant cell and smaller sarcoma cells surrounded by vacuolated cells and blood cells. (350 ×)

Fig 4 Primary tumour cross striated muscle cells with polymorphous nuclei H & E (1000 ×)


1


2


3





small giant cells (Figs 4 and 5) Cells with cross striations were found only in the first 2 passages in small numbers Some loss of differentiation in the tumour as a whole was noted during the ensuing passages In the 11th to 14th passage some capacity to differentiate was still maintained by a tendency to form fascicles of elongated cells (Fig 6), while parts of the tumours were more anaplastic (Fig 7)

The preliminary results of electron microscopy confirmed that the primary tumour consisted of malignant skeletal muscle cells with more or less successful attempts at differentiation (Figs 8 and 9) Indentations of the nuclear surface were typical findings even in highly differentiated cells (Fig 10) A less differentiated giant cell packed with mitochondria is shown in which the most bizarre nuclear shape is conspicuous

DISCUSSION

Rhabdomyosarcomas are rare tumours in man (*Stout & Lattes* 1967) and animals (*Willis* 1967), though they have been reported to occur in several different vertebrate species including fish (see f inst *Fibiger* 1909) A few have been reported to occur in the common laboratory animals, such as spontaneous tumours in the mouse (*Stewart et al* 1959, *Nameroff et al* 1920), and rat (*Bullock & Curtiss* 1922, *Maddock et al* 1962)

Sarcomas with indubitable occurrence of cross-striated muscle cells have been induced by heavy metals (*Heath & Webb* 1967) or virus (*Levy et al* 1969), by MSV (*Moloney* 1966) and also by methylcholantrene (*Clarke* 1969) and cycasin (*Hirono* 1968)

Only two reports on ascites rhabdomyosarcoma in mice are available (*Klein* 1955, *Mellgren et al* 1966), in either case it had been induced by methylcholantrene, but the phenomenon has apparently not been observed in rats

The aetiology of the tumours reported by the author (*Petri* 1968) is not known. They have now been reproduced in a new series of rats under the same conditions, though with a somewhat shorter average latent period, and they are therefore specifically related to some factor in the growth and regression of the Yoshida ascites sarcoma continuously infected by *Nosema cuniculi* Though some authors believe that these tumours were etiologically related to infection with microsporida (*Reznik et al* 1970), this has not yet been clarified A number of transplanted tumours have been found to harbour oncogenic viruses (*Schmidt* 1955) and, if cell free tumour material were injected into animals of the same species, tumours would appear which were unrelated to the original tumours, and be mainly of bone marrow or lymphatic origin This may be the case also with the rats surviving after regression of the Nosema infected Yoshida ascites sarcoma Investigations concerning a possible virus aetiology of the rhabdomyosarcoma and the part played by Nosema are in progress

The loss of differentiation in the present tumour during transplantation is seen to occur in most transplanted tumours (*Stewart et al* 1959) though, until the 13th passage, some capacity of differentiation is retained The number of reports on transplanted rhabdomyosarcomas in rats is small (*Bullock & Curtiss* 1922, *Maddock et al* 1962) The tumours discussed in both publications also contained

---

Fig 5 Another field from the same tumour Giant cells and small, less differentiated cells H & E (140 ×)

Fig 6 Transplanted sarcoma, 14th passage, solid subcutaneous tumour Cross-sectioning bundles of elongated cells H & E (140 ×)

F [illegible] solid [illegible] res [illegible] nast [illegible] equent finding in the present series of tumours Toluidine blue (350 ×)

Fig 8 Electron micrograph of highly differentiated muscle cell from the primary tumour with smaller and larger nuclear indentations. Some irregularity in the organization of myofilaments and sarcoplasmic reticulum will be noted (10 000 ×)



cartilage They behaved differently in the course of continued transplantations in that one became more anaplastic while the other, even after 18 passages, maintained metastasizing muscle tissue with cross striations The present tumour did not give rise to distant metastates after 6 transplantations, neither in the form of a solid nor as an ascitic tumour, but the latter showed vigorous local spread in the abdominal cavity, including the spleen, the pancreas and, occasionally, the liver

The classification of the primary tumour as a rhabdomyosarcoma was based on the occurrence of large numbers of cross striated muscle cells, though with classical cytological features of malignancy, such as hyperchromatic nuclei and extreme nuclear pleomorphism The degree of differentiation, however, varied within rather wide limits, and areas with no obvious resemblance to muscle tissue were found In agreement with the current classification of tumours, such areas can be considered anaplastic muscle cells, as highly differentiated mesenchymal cells of other types were not found This was observed also in the remainder of the present series and in the series reported previously (*Petri* 1968)

Although the classification of tumours of low differentiation naturally is uncertain, it seems reasonable to accept the present transplanted tumour as a rhabdomyosarcoma The ascitic tumour cell which is rich in RNA upon subcutaneous transplantation forms differentiated cells in bundles which may be considered abortive attempts at formation of muscle tissue

---

Fig 9 Electron micrograph of part of a less differentiated muscle cell from the primary tumour with haphazard arrangement of myofilaments and irregularly formed Z discs (Z) The origin of the dense areas closely applied to narrow, closed cisternae is uncertain, but many represent an abortive formation of a transverse tubular system (D) (21,000 ×)

Fig 10 Electron micrograph of a giant cell with a monstrous nuclear shape from the primary tumour The cytoplasm is dominated by closely packed mitochondria (5 000 ×)

The tumour grows vigorously *in vitro* (*Petri* unpublished data)

REFERENCES

Bullock, F D & *Curtis, M R* A transplantable metastasizing chondro-rhabdo-myosarcoma of the rat J Cancer Res *7* 195–207, 1922

Clarke, M A The fine structure of methylcholantrene induced tumors in mice Cancer *24* 147–157, 1969

Fibiger, J Ein Rhabdomyom bei einem Kabljau

mary rhabdomyosarcoma induced in the rat by cobalt, nickel and cadmium Brit J Cancer *21* 768–779, 1967

Hirono, I, *Laquer, G L* & *Spit., M* Transplantability of cycasin induced tumors in rats, with emphasis on nephroblastomas. J nat Cancer Inst *40* 1011–1025, 1968

Klein, G Comparative studies of mouse tumors with respect to their capacity for growth as 'ascites tumors' and their nucleic acid content per cell Exp Cell Res *2* 518–573, 1951

Levy, B M, *Taylor, A C*, *Hampton, S* & *Thoma, G W* Tumors of the marmoset produced by Rous sarcoma virus Cancer Res. *29* 2237–2248, 1969

Maddock, C L, *Kury, L* & *Riley, A* A transplantable metastasizing rhabdomyosarcoma (mesenchymoma) in the W/Fu rat. Cancer Res. *22* 291 293 1962

Mellgren, J, *Bartholdsson, E*, *Boerjd, B* & *Norrby, K* A spontaneously metastasizing 20 methyl cholantrene induced rhabdomyosarcoma and its transformation to ascites form in the CBA mouse Acta path microbiol scand *68* 535–546, 1966

Moloney, J B A virus-induced rhabdomyosarcoma of mice Nat Cancer Inst Monogr *22* 139–142, 1966

Nameroff, M A, *Reznik, M*, *Anderson, P* & *Hansen, J L* Differentiation and control of mitosis in a skeletal muscle tumor Cancer Res *30* 596–600, 1970

Petri, M A cytolytic parasite in the cells of transplantable, malignant tumours Nature (Lond) *205* 302, 1965

Petri, M The occurrence of *Nosema cuniculi* (*Encephalitozoon cuniculi*) in the cells of transplantable, malignant tumours and its effect upon tumour and host Acta path microbiol scand *66* 13 30, 1966

Petri, M High incidence of intraperitoneal rhabdomyosarcomas in rats after growth and regression of *Nosema cuniculi* infected Yoshida ascites

sarcoma. Acta path microbiol scand *73* 1 12, 1968
Petri, M Studies on *Nosema cuniculi* found in transplantable ascites tumours with a survey of microsporidiosis in mammals Acta path micro biol scand, Suppl 204, 1969
Reznik, M, Nameroff, M A & Hansen, J L Ul trastructure of a transplantable murine rhabdo myosarcoma. Cancer Res *30* 601–610, 1970
Schmidt, F Über Filtratversuche mit Mausetrans plantationstumoren Z Krebsforsch *60* 445–455, 1955
Sparck, J V Nogle retningslinier for indavl af forsøgsdyr Publ by Dansk Selskab for Cancer forskning, 1968
Stewart, H L, Snell, K C, Dunham, L J & Schlyen, S M Transplantable and transmissible tumors of animals Armed Forces Institute of Pathology, Washington 25, D C, 1959
Stout, A P & Lattes, R Tumors of the soft tissues. Armed Forces Institute of Pathology, Washington 25, D C, 1966
Willis, R A Pathology of tumours (4 ed) Butterworths, London, 1967, p 768

Acta path microbiol scand Section A 81, 453–456, 1973

CELLULAR REACTION TO GESTATIONAL CHORIOCARCINOMA AND INVASIVE MOLE

BENT MOGENSEN and STEEN OLSEN

The Institute of Cancer Research, the Danish Cancer Society, Radiumstationen, and the University Institute of Pathology, Aarhus Kommunehospital, Aarhus, Denmark

Cellular reaction to primary and metastatic gestational choriocarcinoma (CHC) was observed in histological sections from all but one of the 23 patients included in this series The reaction was slight in 8, moderate in 5 and marked in 9 The infiltrates consisted of lymphocytes and plasma cells A statistically significant correlation ($p < 0.05$) was demonstrated between the intensity of the reaction and the recovery of the patients In 8 cases of primary invasive mole (IM) a similar reaction was observed All these patients recovered None of the 31 patients had had any treatment before the removal of the histological specimens examined in this study

In the literature on gestational choriocarcinoma (CHC) there is little reference to a cellular reaction around the tumour Some investigators who describe many histological details do not mention a reaction at all, others specifically note the absence of a reaction, and others again describe round cells peritumourally without discussing their possible significance (for references, see 2) The first formal investigation of this histological feature seems to have been carried out by *Elston* (2), who observed a cellular accumulation around CHC in sections from 35 of 38 patients. He also noted that patients with the greater degree of leucocytic infiltration responded more favourably to treatment Later, *Park* (8) reported on 50 cases of CHC, and his findings were largely in agreement with the results described by *Elston*

Only one analysis of cellular reaction to invasive mole (IM) seems to have been published *Park* (7) studied 43 examples of this tumour and concluded that it was quite exceptional for lymphocytes to be less than abundant around the hyperplastic trophoblast

In view of our interest in the immunology of post gestational trophoblastic tumours (6), all available histological material from the cases diagnosed in this country from 1940 up to the end of 1970 was investigated, but in contrast to previous investigations (2 7, 8) only material from untreated patients was included in the present study It is the purpose of this paper to present the results of our study to correlate the degree of cellular response to the fate of the patient, and to discuss some immunological aspects of relevance to the histological picture

Received 26 1 73 Accepted 26 1 73

Request for reprints should be addressed to Bent Mogensen, the Institute of Cancer Research, Radiumstationen DK-8000 Aarhus C Denmark

MATERIAL AND METHODS

Histological specimens from 38 cases of trophoblastic neoplasms were available for the purpose of this investigation Twenty eight of the tumours

were classified as CHC and 10 as IM The criteria for inclusion of the specimens were (a) no cancer chemotherapy or irradiation prior to the removal of the tumour, (b) absence of neutrophils around the tumour, and (c) direct contact of the tumour with the living host tissue On this basis 5 cases of CHC and 2 of IM were excluded, and so were the metastases from 5 CHC patients whose primary tumours were included in the study, i e, only tissue unmodified by treatment or complicating acute inflammation was investigated

Thus, in the CHC group 19 primary tumours (uterus 18, Fallopian tube, 1) fulfilled the criteria for inclusion and in one of these cases a vaginal metastasis was also available In 4 cases, only metastases (vagina, lung liver and kidney) were at our disposal In 2 of them no tumour was found by careful investigation of the hysterectomy specimen one uterus was not investigated and in the last case a tumour of the myometrium had been demonstrated both grossly and histologically but the specimens were no longer available The 8 cases of IM were all primary myometrial tumours The histological material was obtained at operation in 29 cases and by autopsy in 2 (both CHC)

The classification of all the tumours but one was described in a recent paper (4) The same criteria were applied by the investigators cited in this paper (2 7 8)

Paraffin embedded material stained with haematoxylin eosin and methylgreen pyronin was studied

The cellular reaction around the tumour was graded as nil (—) slight (+) moderate (++), and marked (+++) and the percentages of the various types of mononuclear cells entering into the reaction were estimated semi quantitatively Further it was recorded whether the cells formed a definite band or some parts of the tumour were left without any reaction To ensure objective evaluation slide numbers and names were masked since the case histories of the patients were known at the time of the study

RESULTS

The cellular reaction to CHC—primary as well as metastatic—and to IM was identical as regards the localization and the type of the mononuclear leucocytes In both forms of tumours, the leucocytes were situated in the normal tissue adjacent to the invading trophoblast Sometimes a zone of normal tissue of varying width separated the cellular reaction from the trophoblast but most frequently the reaction was in direct contact with the surface of the tumour Within the living solid tumour tissue, neither mono- nor polymorphonuclear cells were observed, in contrast to the necrotic areas in which the latter type of cell was usually present Numerically the small lymphocyte was the predominant cell type in all reactions, large lymphocytes being present only in small numbers Plasma cells were constantly present. Generally, they constituted only a small percentage, but in some specimens their number reached 10-20 per cent of the total population Finally, in all the slides, cells with an appearance of transitional stages between lymphocytes and plasma cells were observed, and many of them had pyroninophilic cytoplasm

When mononuclear cells occurred in large numbers (+++), they formed a definite, continuous band around the trophoblast in 4 cases and the band was discontinuous in 5 leaving some part of the tumour without any reaction In slight and moderate reactions (+, ++), a continuous band was never observed In the slight reactions, the cells were typically located around small venules In specimens with marked reaction this feature was less conspicuous but small vessels could often be identified centrally in the most densely packed areas of the cell aggregates

A cellular reaction to CHC was found in 22 of the 23 patients The reaction was slight in 8 moderate in 5, and marked in 9 (Table 1) Eleven of the CHC patients died of generalized disease, and 12 including 6 with metastases, recovered In order to see whether any correlation existed between the intensity of cellular reaction and the course of the malignant disease, these 2 factors were related to each other (Table 2) Owing to the small number of patients, no or slight reactions were grouped together (mild reaction), and so were moderate and marked (severe reaction) Nine patients showed mild reaction including 7 who died of generalized disease as compared with 4 deaths among 14 patients who had severe reactions Application of Fisher s exact test showed that the distribution of these figures was statistically significant at the 5 per cent level ($p = 0.029$) The survivors are all well at the time of writing

TABLE 1 *The Intensity of the Cellular Reaction Surrounding the Tumours in 23 Choriocarcinoma Patients*

Site	Nil (—)	Slight (+)	Moderate (++)	Marked (+++)	Total
Primary	0	7	4	7	18
Primary and metastatic	0	0	1*	0	1
Metastatic	1*	1	0	2	4
Total	1	8	5	9	23

* One of these patients had 4 the other 2 deposits of tumour tissue, both showed the same intensity of *reaction nil and moderate, respectively to the various deposits*

TABLE 2 *The Intensity of Cellular Reaction Related to the Course of the Disease in the 23 Choriocarcinoma Patients*

	Mild (—, +)	Severe (++, +++)	Total
Died	7	4	11
Recovered	2	10	12
Total	9	14	23

$p = 0.029$ (Fisher's exact test)

the observation periods being more than 5 years in 11 cases and less in one

Reactions were observed in all the IM patients, the cellular infiltrate was slight in 1, moderate in 4, and marked in 3 No definite band was observed in any case Moderate eosinophilia was noted together with the mononuclear cells in the specimens from one patient All the patients are well today, with observation periods ranging from 3 to 28 years

DISCUSSION

As the histological material studied was obtained from patients who had *not received* any previous treatment, the cellular reaction which was observed in all cases but one must be a spontaneous biological phenomenon without any possible interference from local (irradiation) or systemic (chemotherapy) factors. It seems unlikely that the curettage which had been performed prior to operation in many of the cases had influenced the reaction as the uterine specimens studied were excised from the depth of the myometrium at an appropriate distance from the cavity The infiltrate was made up of lymphocytes and plasma cells, which points towards the possibility that it represents an immunological reaction against the tumour tissue

In the present study, the mononuclear cells formed a definite band in 17 per cent (4/23) of the CHC cases, as compared with 43 per cent (16/37) of the cases in *Elston's* series (2) This discrepancy may have some relation to the treatment given to some (most?) of the patients in *Elston's* series before the histological material was removed

A statistically significant association was observed between the degree of cellular reaction and the prognoses of the CHC patients, which is in agreement with previous results (2) An intense cellular infiltration thus seems to be of fundamental importance in the fate of CHC patients. To us this finding is ama-

zing in view of the many different regimens of treatment which our patients—originating from a period of 31 years (5)—were given *after* the excision of the histological material used in this study. From a theoretical point of view, it would be of great interest if the results could be confirmed in larger series of patients. In medical practice, the potential prognostic value seems to be of decreasing importance because, today, cancer chemotherapy is the treatment of choice in this type of tumour, and attempts to assess the prognosis by means of biopsies would hardly be in the best interests of the patients.

As assessed by the intensity of cellular reaction, the prognosis differed from the course of the disease in the 4 patients with severe reaction who died. However, their mean survival time from the termination of the pregnancy antecedent to diagnosis was 52.5 months, as compared with only 8.3 months in the 7 patients with mild reaction (Table 2). This difference was not due to early diagnosis and treatment. On an average, the diagnosis was established 14.8 months after the preceding pregnancy in the former group, as compared with 5.7 months in the latter. Two more patients diverged from the prognosis. Both were from the group with mild reaction, both had metastases, and both recovered. A common feature in their treatment was moderate cancer chemotherapy (total doses of 160 and 210 mg Methotrexate were given in 2 and 5 series, respectively).

All the 8 IM patients had cellular reactions peritumourally, and they all recovered. Owing to its invasive growth and capability of metastatic spread, the disease is by definition a malignancy, but in general it runs a benign clinical course. Thus all 23 cases—7 of them with metastases—which were diagnosed in Denmark from 1940 up to the end of 1971 recovered.

At present it is uncertain whether the possible immunogenicity of trophoblastic neoplasms is provoked by tumour-specific, organ-specific or transplantation antigens. No evidence seems to exist in favour of the first possibility. Recently, *Krieg* (3) described an organ-specific antigen in normal trophoblast of human term placenta, but so far there is no evidence suggesting that this antigen is also present in malignant trophoblast. The unique position of trophoblastic neoplasms being allografts opens up the possibility that transplantation antigens—of paternal origin—may play a role. This possibility was long considered unlikely, but increasing support of this assumption has been obtained during recent years (see 6). The cellular composition and the localization around small venules of the reaction described in this study are highly reminiscent of some features of the allograft reaction observed, for example, in the renal allograft. However, in the latter situation the reaction occurs in *the graft* in contrast to trophoblastic tumours, in which the infiltrate is observed in the recipient's tissue surrounding the tumour. Presumably this difference is due to the avascularity of the trophoblastic tumours—an assumption which is supported by the observation of a similar immunopathological pattern in another avascular graft: the transplanted, incompatible cornea (1).

REFERENCES

1. *Ehlers, N. & Ahrons, S.*: Corneal transplantation and histocompatibility. Acta ophthal. (Kbh.) *49*: 513–527, 1971.
2. *Elston, C. W.*: Cellular reaction to choriocarcinoma. J. Pathol. *97*: 261–268, 1969.
3. *Krieg, H.*: Antigenicity of human chorionic villous tissue. Int. J. Gynec. Obstet. *10*: 144–155, 1972.
4. *Mogensen, B. & Olsen, S.*: Gestational choriocarcinoma in Denmark 1940–1969. A reappraisal based on modern histologic criteria. Acta obstet. gynec. scand. *51*: 63–69, 1972.
5. *Mogensen, B.*: Invasive mole and gestational choriocarcinoma in Denmark 1940–1969. Biological and clinical aspects. (to be published in Acta obstet. gynec. scand.)
6. *Mogensen, B. & Kissmeyer Nielsen, F.*: Invasive mole, gestational choriocarcinoma and transplantation antigens. Ser. Haematol. V *5*: 22–43, 1972.
7. *Park, W. W.*: Choriocarcinoma. A study of its [illegible]
8. [illegible] Pathology. William Heinemann Medical Books Ltd., London 1971, p. 114–118.

Acta path microbiol scand Section A 81, 457–460, 1973

ASBESTOS BODIES AND PLEURAL PLAQUES IN HUMAN LUNGS AT NECROPSY

I Hägerstrand and B Seifert

University Department of Pathology, General Hospital Malmo, Sweden

Ninety seven, largely consecutive, subjects (59 men and 38 women) in Malmo, a coast town of 250 000 inhabitants in south Sweden, were examined post mortem for pleural plaques and asbestos bodies of the lungs Two sections and one smear from each lung were examined Asbestos bodies were found in 47 (32 men and 15 women) and pleural plaques in 29 (26 men and 3 women) Sixteen out of the 32 men with asbestos bodies had also pleural plaques In 7 subjects, all men more than 20 asbestos bodies were found, four of these had pleural plaques

The frequency of asbestos bodies in lungs of subjects with malignant mesothelioma autopsied at the Institute of Pathology, Malmo, Sweden, between 1957 and 1966 has been reported earlier (8) The correlation between asbestos bodies in lungs and malignant mesothelioma was not as close as that found in other large towns in the world It was therefore considered desirable to find out the frequency of asbestos bodies in the general population of Malmo compared with that in other large towns (1-7, 9, 11-15, 17 22) This paper is based on 97 largely consecutive autopsies performed in the autumn of 1967 Smears and sections were examined for asbestos bodies in the lungs A search was also made for pleural plaques. It has been claimed that pleural fibrocalcific plaques can be caused by asbestos (7, 11, 12)

MATERIAL AND METHODS

Ninety seven subjects (59 men, 38 women, children were excluded) were examined for pleural plaques, their frequency, extent and sites One smear (slides 75 × 25 mm) and one tissue block (about 1 5 × 1 5 cm² after fixation) were obtained from the lower lobe of each lung Two sections (30μ) were cut from each tissue block The sections and smears were studied unstained, and the asbestos bodies were counted (8) There were 4 cases with pulmonary carcinoma and one with malignant mesothelioma, but no case with asbestosis. The mean age of the men was 72, (range 38 92) that of women 71 2, (range 30-93) The occupations of the subjects were noted if available from the case records

Received 19 x.72 Accepted 29 i 73

Requests for reprints should be addressed to *Inga Hagerstrand*, University Department of Pathology, General Hospital, 214 01 Malmo, Sweden

RESULTS

Asbestos bodies were found in sections and/or smears from 47 (48 4 per cent) subjects, 32 (54 2 per cent) of whom were men and 15 (39 4 per cent) women Asbestos bodies were found in smears in 22 (20 6 per cent) cases (15 men and 7 women) and in sections in 43 (44 3 per cent) cases (31 men and 12 women) Asbestos bodies were found only in smears in 4 cases and only in sections in 25 More than 20 asbestos bodies were found in 7 subjects, if sections and smears were taken together, in 5 if only sections were examined All the 7 subjects were men Seventeen (36 2 per cent) out of the 47 subjects with asbestos bodies had pleural plaques, sixteen of these

were men, i e 50 per cent of the men with asbestos bodies had pleural plaques Four out of 7 men with more than 20 asbestos bodies had pleural plaques

Pleural plaques were found in 29 (30 per cent) subjects, 26 (44 per cent) men and 3 (7 5 per cent) women Asbestos bodies were found in smears and/or sections of the lungs in 17 (58 6 per cent) among the 29 cases In 18 out of the 29 cases the plaques were bilateral They were multiple of size varying from some cm up to some dm diameter If small, they were found near the vertebral column above the diaphragm, otherwise, also in other parts of the parietal pleural surface and especially on the lateral surfaces The apices were free from plaques

Two of the 4 subjects with pulmonary carcinoma had a few asbestos bodies, but no plaques The subject with malignant mesothelioma showed neither bodies nor plaques

Informations about occupations were insufficient, especially as regards the women The 7 men with more than 20 asbestos bodies had occupations where asbestos pollution of the air might occur (painter, cooper, cement worker, fitter, stockroom man, welder, chief engineer) However, no subject had had an occupation in which asbestos is a known hazard

DISCUSSION

The frequencies of asbestos bodies found in different materials are not strictly comparable owing to differences in the techniques used Most workers have used smears, while a few used sections We had quite different result with these two techniques and the section technique was the best one in our hands *Meurman* (11) used only this technique and found among 264 examined cases 57 6 per cent with asbestos bodies (60 1 per cent in men, 54 3 per cent in women) *Hourihane* (7) reported a series from London of 115 cases with asbestos bodies in 24 5 per cent, using lung sections *Gibson* (9) reported from Glasgow 41 5 per cent cases with asbestos bodies in lung sections obtained from 600 cases examined Irrespective of the methods used it is obvious that pulmonary asbestos bodies are common in inhabitants of large towns all over the world (Table 1) The researches by *Chang Hyun Um* from London (4, Table 1) which showed an increasing frequency of asbestos bodies in the period from 1936 to 1966 nicely reflects the increasing asbestos air pollution during the last decades In the series from Perugia in Italy asbestos bodies—only 2—were found in one out of 109 cases examined and thus, it differs strikingly from the other series (14, table 1) According to the authors, Perugia is not an industrial city and the air is not notably polluted No case of mesothelioma has ever been seen in that region —It is known from *Pooley's* (16) re examination of the mesotheliomas in Malmo that about 90 per cent of all cases with asbestos bodies also have asbestos fibres, and that the bodies are therefore due at least mainly, to exposure to asbestos Malmo is a coast town (250,000 inhabitants), it has no asbestos mines, and workers in the shipbuilding, insulation and building industries are the ones who are most heavily exposed to asbestos Any accumulation of these types of workers was not observed in the series which seems to represent an ordinary population of Malmo It is an important problem to solve whether or not the urban air pollution of asbestos today is carcinogenic and it seems essential to follow the frequency of mesothelioma in different parts of the world *Pooley's* (16) re examination of the cases of mesothelioma in Malmo revealed a difference between the frequency of asbestos fibres in the tumour series and in the control series (82 per cent versus 53 per cent) larger than that observed in the primary examination (53 per cent versus 35 per cent) The fibres and bodies were also more often abundant in the tumour series Thus, also the Malmo mesothelioma material suggests a correlation between asbestos exposure and the tumour However, the occupations of the subjects in the series did not indicate an exposure to asbestos diverging from that in the normal population of Malmo (8)

TABLE 1 *Survey of Autoptical Reports of the Frequency of Pleural Plaques and the Frequency of Asbestos Bodies in Lung Smears*

Place Year / reference number	Numbers men	Numbers women	Numbers total	Pleural plaques	Asbestos bodies smears men	Asbestos bodies smears women	Asbestos bodies smears total
Town, 1960 th Africa (21)	306	194	500		30 4 %	20 %	26 4 %
Miami, USA, 1961 (21)	304	195	500		31 6 %	20 4 %	27 2 %
ttsburg, USA, 965 (3)	53	47	100		47 %	34 %	41 %
treal, Canada, 1' 5 (1)	56	44	100		57 %	36 %	48 %
Glasgow, Scotland, 1967 (18)	62	38	100	16 % (= in 13 men 11 with asbestos bodies)	37 %	0 %	23 %
Milano, Italy, 1967 (6)	64	36	100	0 % (= in 65 examined cases no plaques)	54 %	44 %	51 %
en, East Germany, (20)			250	4 % (= 10 cases, 6 with asbestos bodies)			43 2 %
upon Tyne England, 1968 (2)	196	115	311		25 5 %	11 3 %	20 3 %
Jerusalem, 1968 (17)			100		29 1 %	22 2 %	26 %
Michigan, USA, 1969 (5)	66	34	100		23 %	11 8 %	18 %
Perugia, Italy, 1969 (14)	63	46	109		1 5 %	0	1 %
Melbourne, Australia, 1969 (22)	138	62	200	0 5 % (1 case)	44 2 %	41 9 %	43 5 %
					digest of lung tissue		
chwerin, Germany, 971 (13)	121	113	234		11 %	8 %	9 %
					digest of lung tissue		
ajevo, Yogoslavia, 1971 (15)	55	45	100		52 7 %	20 %	38 %
London 1936	82	45	127				0
1946	61	39	100		4 9 %	0	3 %
1956	51	49	100		15 7 %	12 2 %	14 %
1966 (4)	55	45	100		18 1 %	22 2 %	20 %
London 1964			381	4 2 %			
1965 (7)			134	11 2 %			
Finland (3 different s) 1966 (11)			438	39 3 % (27 % bilateral plaques, among these 71 5 % in men)			
Glasgow, Scotland, 1971 (19)			334 41	12 3 % = 41 cases	40	1	85 3 %

Meurman's morphological description of the pleural plaques (10, 11) is in good agreement with the appearance of those in the Malmo series The frequency of the plaques is roughly as high in the Malmo series as in *Meurman's* series from Finland, and the plaques were bilateral equally often in the two series (62 per cent in Malmo, 70 per cent in Finland) They were much more frequent in men than in women The high frequency of the plaques in Finland and Malmo is remarkable, it is at least twice the frequency reported from other places—Glasgow, London Milano, Dresden, Melbourne (Table 1)

Meurman found a good correlation between the occurrence of pleural plaques and asbestos bodies Fifty per cent of the men with asbestos bodies in the Malmo series also showed pleural plaques, the corresponding figure being 37 per cent for men without asbestos bodies *Meurman* considered some unknown pathogenic factors other than asbestos as at least contributory to the plaques (12) One radiological report from Borås in Sweden is available which showed a high frequency of asbestos exposure among 42 men with pleural plaques (10) Of course, the plaques might be caused by asbestos whether or not co existing asbestos bodies were in evidence Taking this possibility into consideration, 70 per cent of the male and 45 per cent of the female population of Malmo would have morphological signs of the fact that they were living in an asbestos polluted air

REFERENCES

1 *Anjilvel, L* & *Thurlbeck, W M* The incidence of asbestos bodies in the lungs at random necropsies in montreal Canad Med Ass. J *95* 1179 1182, 1966

2 *Ashcroft T* Asbestos bodies in routine necropsies on Tyneside a pathological and social study Brit Med J *1* 614–618, 1968

3 *Cauna, D, Totten, R S* & *Gross P* Asbestos bodies in human lungs at autopsy, J Am Med Ass *192* 371–373 1965

4 *Chang Hyun Um* Study of the secular trend in asbestos bodies in lungs in London 1936 1966 Brit Med J 2 248 252, 1971

5 *Dicke T E* & *Naylor B* Prevalence of asbestos bodies in human lungs at necropsy Diseases of chest *56* 122 125, 1969

6 *Ghezzi, I, Molteni, G* & *Puccetti, U* Asbestos bodies in the lungs of inhabitants of Milan Medicina del Lavoro *58* 223 226 1967

7 *Hourihane, D O B, Lessof, L* & *Richardson, P C* Hyaline and calcified pleural plaques as an index of exposure to asbestos A study of radiological and pathological features of 100 cases with a consideration of epidemiology, Brit Med J *1* 1069–1074, 1966

8 *Hagerstrand, I, Meurman, L* & *Odlund, B* Asbestos bodies in the lungs and mesothelioma, a retrospective examination of a ten year autopsy material, Acta path et microbiol scandinav 72 177–191, 1968

9 *Mac Pherson, P* & *Davidson, J K* Correlation between lung asbestos count at necropsy *and radiological appearances*, Brit Med J *1* 355–357, 1969

10 *Mattsson, S B* & *Ringqvist, T* Pleural plaques and exposure to asbestos, Scand J Resp. Dis Suppl 75, 1970

11 *Meurman, L* Asbestos bodies and pleural plaques in a Finnish series of autopsy cases Acta *path et microbiol scandinav Suppl.* 181 *1966*

12 *Meurman, L O* Pleural fibrocalcific plaques and asbestos exposure Environmental Research *2* 30–46, 1968

13 *Nizze, H* Zur Häufigkeit sogenannter Asbestkorper in menschlichen Lungen Internationales *Archiv fur Arbeitsmedizin 28 71 82 1971*

14 *Peacock, P R, Biancifiori, C* & *Bucciarelli E* Examination of lungs smears for asbestos bodies in 109 consecutive necropsies in Perugia Europ J Cancer *5* 155–158, 1969

15 *Plamenac P, Pikula, B Kahvic, M, Markovic, Z Selak, I* & *Zeger Vidovic, Z* Incidence of asbestos bodies in basal lung smear Acta med ing *25* 325–332 1971

16 *Pooley F D* Asbestos fibre in the lung and mesothelioma A re-examination of the Malmo material To be published

17 *Polliack A* & *Sacks, M I* Prevalence of asbestos bodies in basal lung smears Israel J Med. Sci *4* 223–226 1968

18 *Roberts, G H* Asbestos bodies in lungs at necropsy, J clin Path *20* 570–573, 1967

19 *Roberts G H* The pathology of parietal pleural plaques, J Clin Path *24* 348–353, *1971*

20 *Rottzsch, E* Über das Vorkommen von Asbestkopperchen in Lungen des Routine-Sektionsmaterials Gegenbaurs morphologisches Jahrbuch *111* 265–267, 1967

21 *Thomson, J G* Asbestos and the urban dweller Ann New York Acad Scienc *132* 196–214 1965

22 *Xipell, J M* & *Bhathal P S* Asbestos bodies in lungs an Australian report *Pathology 1* 327 330 1969

Acta path microbiol scand Section A 81, 461-473, 1973

LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS ON GLOMERULAR CHANGES IN CANINE INTERSTITIAL NEPHRITIS

KAI KROHN, PENTTI T JOKELAINEN and MARKUS SANDHOLM

The I Department of Pathology, University of Helsinki, Helsinki, Finland,
from the Cardiovascular- and Immunopathology Laboratory,
The New York Blood Center, New York, from the Department of Anatomy,
New York Medical College, Valhalla, New York,
and from the Department of Medicine, College of Veterinary Medicine, Helsinki, Finland

Light and electron microscopic studies on dogs suffering from chronic interstitial nephritis revealed glomerular lesions characterized by thickening of the glomerular capillary walls and an increase in the mesangium The thickened capillary walls stained strongly with PAS stain and less with silver methenamine stain Electron microscopy revealed the presence of subendothelial and occasional subepithelial electron dense deposits in the thickened and split glomerular basement membrane Degenerative changes were seen in the epithelial as well as in the endothelial cells Similar but milder lesions were seen in clinically healthy old control dogs but not in young healthy dogs The findings emphasize the importance of glomerular lesion in canine interstitial nephritis and supports the hypothesis that an altered immunological mechanism probably related to ageing might be pathogenetic in this disease

Chronic interstitial nephritis in the dog (CIN) occurring frequently in older dogs of both sexes, is histologically characterized by the presence of a lymphocytic and plasmacellular infiltration in the cortical interstitium of the affected kidneys (11, 13) The tubuli *especially in cases of advanced lesions* are dilated and atrophic and the glomeruli show varying degrees of histological alterations (1) Although the cause of the disease is unknown, the alterations have been attributed to a chronic infection with leptospirae this assumption is based on the fact that the interstitial changes are similar to those seen in acute interstitial nephritis of the dog which accompanies acute leptospiral infection and that this acute form of the disease sometimes develops into a chronic interstitial nephritis In the previous part of this study (11) we demonstrated that immunoglobulin IgG as well as complement component C3 were deposited along the capillary walls of affected glomeruli in CIN, and that antibodies eluted from the affected kidneys did not have anti-leptospiral activity but reacted against the nephritic glomeruli and not against any normal canine tissue These studies did not support the assumption of a leptospiral aetiology in CIN and indicated that antigen-antibody complexes were deposited in the glomeruli of

Received 6 x 72 Accepted 9 ii 73

Requests for reprints should be addressed to Dr *Kai Krohn*, Haartmanstreet 3 00290 Helsinki 29, Finland

affected kidneys and could thus be of pathogenic significance to the disease We now report light microscopic and electron microscopic findings in CIN Our results indicate that the characteristic glomerular lesion in CIN is of membranoproliferative type and that electron dense deposits that could represent antigen-antibody complexes are present in the thickened glomerular basement membranes (GBM) of the affected glomeruli

MATERIAL AND METHODS

Study Group

Twenty four dogs of different breeds were studied Twelve were selected because of a clinical diagnosis of chronic interstitial nephritis, based on a history of polyuria, polydipsia, malaise and occasional vomiting Slight proteinuria and elevated blood urea nitrogen (BUN) were present in all these dogs The age of the dogs, when known, varied from 6 to 13 years Eight clinically healthy dogs of ages varying from 6 to 17 years and four similarly healthy one year-old dogs served as a control group

Each dog was anaesthetized by intravenous sodium thiopenthone (Intraval®) The kidneys were removed and the dog sacrificed by bleeding or by an extensive dose of the anaesthetic Other parenchymal organs were then removed for the study On some occasions a biopsy was taken from the kidneys for electron microscopic study while the circulation of the kidneys was *not severed*

Tissue Preparations

Electron microscopy Five to ten tissue pieces measuring about 1 mm^3 were taken from different parts of the cortex from each kidney either before the kidney was removed from the anaesthetized dog or immediately after The tissue samples were fixed for 50 to 60 minutes in 2 5 per cent glutaraldehyde buffered to pH 7 4 with 0 1 M phosphate buffer After fixation the samples were washed with cold buffer postfixed for one hour with 1 per cent osmic acid dehydrated in graded alcohols and embedded in Epon 812 The sections were stained with saturated or 5 per cent uranyl acetate followed by staining with lead citrate and examined with a Siemens Al Elmiskope electron microscope

Light microscopy Tissue samples from the kidneys as well as from other parenchymal organs were fixed in neutral formalin with or without prior fixation with Bouin s solution and embedded in paraffin The sections were stained with HE PAS Masson s trichrome stain and Jones periodic acid and silver methenamine stain

Immunological studies Cryostat sections were prepared from kidneys and stained with fluorescein labelled anti-canine IgG or with anti canine immunoglobulin sera as described earlier (11)

RESULTS

Light Microscopic Findings

Control dogs No pathological lesions were seen in the kidneys of the one year old control dogs There was no fibrosis or inflammatory cell infiltration in the interstitium. The capillary walls in the glomeruli were thin and the lumina widely open (Fig 1) In the older control dogs the glomerular basement membranes (GBM) were thickened as demonstrated by PAS and silver methenamine staining (Figs 2 and 3) There was occasional interstitial fibrosis in the cortex, but no inflammatory cell infiltration was present The tubular epithelium appeared to be normal

Nephritic dogs All the dogs with clinical signs of chronic interstitial nephritis had pathological changes in the kidneys In one case these were typical of a purulent pyelonephritis and this case was consequently excluded from further studies In the remaining cases, lymphocytic and plasmacellular infiltration of different grades were observed in the cortical and sometimes also in the medullary interstitium of the kidneys Based on the degree of glomerular involvement the material was divided into three groups those with mild

Fig 1 Glomerulus of a young control dog without any pathological changes PAS 300 ×

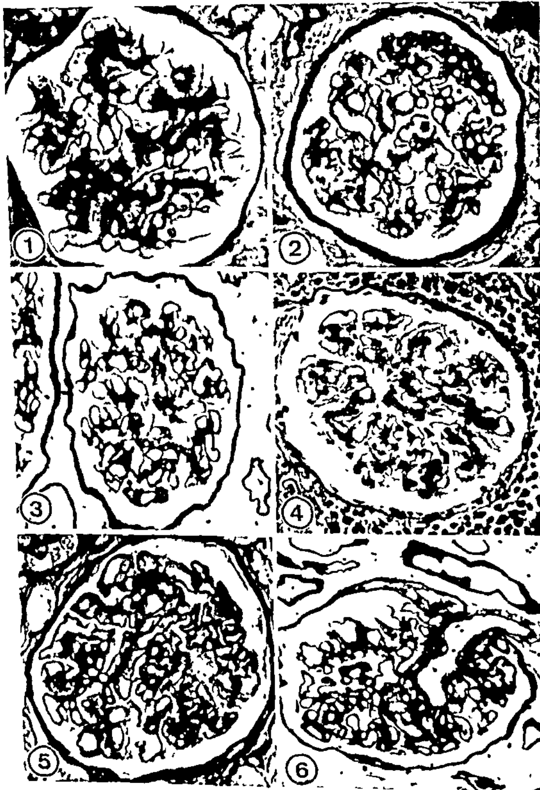
Fig 2 Glomerulus from an older control dog with slightly thickened capillary walls PAS 300 ×

Fig 3 Glomerulus from an older control dog showing increase of mesangial matrix but capillary walls of normal thickness. Silver methenamine stain 300 ×

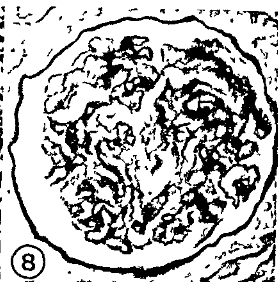
Fig 4 Glomerulus from a nephritic dog showing mild changes There is an increase in the mesangial matrix and the capillary walls appear to be slightly thickened Masson s Trichrome stain. 300 ×

Fig 5 Nephritic dog with mild glomerular changes. The glomerular capillary walls are thickened in PAS stained preparation 300 ×

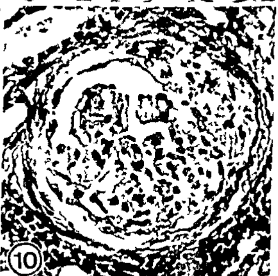
Fig 6 Same case as in Figs 4 and 5, increase in mesangial matrix Silver methenamine stain 300 ×

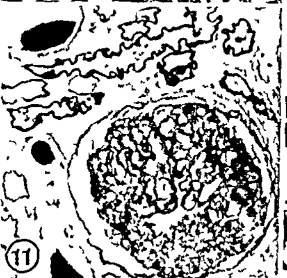

1
2
3
4
5
6


7


8


9


10


11


12

moderate, or advanced glomerular changes

Mild glomerular changes were observed in four dogs Although many of the glomeruli in these cases were similar to those observed in the older control dogs, the majority of glomeruli showed an increase in the mesangial matrix as well as in the mesangial cellularity (Fig 4) In addition the GBM were slightly thicker than in the older control dogs, this alteration being more pronounced in the preparations stained with PAS than in those stained with silver methenamine (Figs 5 and 6) Occasional glomeruli showed slight segmentation of the capillary tuft Neither epithelial adhesions nor pericapsular fibrosis was observed

Lymphocytic and plasmacellular infiltration were observed in the cortical interstitium in only a few scattered areas in these cases The convoluted tubuli adjacent to these infiltrations had slightly degenerated epithelia, but neither tubular dilatations nor atrophy of the tubular epithelium was observed Interstitial fibrosis was mild or totally absent

Fig 7 Glomerulus from nephritic kidney Moderate glomerular changes The glomerular tuft is segmented and there is a clear increase in mesangium The capillary walls are also clearly thickened Wire loop lesions are seen at eight and nine Masson s Trichrome stain 300 ×

Fig 8 Same case as in Fig 7 PAS stain showing thickened capillary walls 300 ×

Fig 9 A case of advanced glomerular lesions showing several partly or totally hyalinized glomeruli advanced fibrosis and tubular dilatation There is a moderate interstitial infiltration with lymphocytes and with plasma cells HE 60 ×

Fig 10 A higher magnification of a glomerulus seen in Fig 9, showing crescent formation and almost total occlusion of the glomerular capillaries. 300 ×

Fig 11 Same case as in Figs. 9 and 10 PAS stain showing positive staining in the mesangium and in the capillary walls 300 ×

Fig 12 Silver methenamine staining of a glomerulus from the same case as in Figs 9 10 and 11, showing staining of the mesangium to some extent but negative staining reaction in the capillary walls 300 ×

Moderate glomerular changes were present in five dogs in which all glomeruli showed some degree of pathological alterations although the extent of the lesions varied The glomerular tuft was usually slightly segmented and there was a clear, although not pronounced, increase in the mesangial matrix as well as in the mesangial cells (Fig 7) In PAS stained preparations (Fig 8) the capillary walls were clearly thickened, and the PAS positive material seemed to be increased on the endothelial side of the GBM In silver methenamine stained preparations, the GBM was only slightly thicker than in the glomeruli of the former group or of the control dogs Occasional adhesions between parietal and visceral epithelia were observed, and in some, glomeruli proliferation of the visceral epithelial cells had led to a crescent formation

In the kidneys with moderate glomerular changes a pronounced infiltration of the interstitium with mononuclear cells was always present Both lymphocytes and plasmacells were seen, the latter being the prominent cell type Usually more than half of the cortical area was involved by this inflammatory process and in some cases the infiltration often extended into the medulla and even into the papillary tip There was moderate interstitial fibrosis and the tubular basement membranes were clearly thickened presenting an irregular contour Tubular dilatations were present, especially in the proximal convoluted tubuli, although some of the distal tubuli also showed this change The epithelium of the dilated tubuli was flattened and the cells were degenerated, sometimes showing intracytoplasmic inclusions Cell debris proteinaceous casts and occasional granulocytes were present in the tubular lumina

Advanced glomerular changes were seen in two dogs In these cases the glomeruli showed a pronounced increase in PAS positive material in the matrix as well as thickening of the capillary walls (Fig 11) Using silver methenamine staining, the GBM did not show as marked a thickening (Fig 12) In many glomeruli the increase in the mesangial matrix together with thickening of the capil-

TABLE 1 *Occurrence of Electron Microscopic Lesions in Control Dogs and in Dogs Suffering from Chronic Interstitial Nephritis*

	Nephritic dogs		Control dogs	
	With mild light microscopic changes (4 cases)	With moderate light microscopic changes (8 cases)	One year old dogs (4 cases)	6- to 17 year-old dogs (8 cases)
Epithelial cell degeneration	+	+ + +	—	—
Epithelial deposits	+ +	+ + +	—	+
Thickening and splitting of glomerular basement membrane	+ +	+ + +	—	+
Subendothelial deposits in the glomerular basement membrane	+ +	+ + +	—	+
Lipid droplets in the glomerular basement membrane	+ +	+ +	—	+
Endothelial cell degeneration	+	+ + +	—	—
Proliferation of endothelial and mesangial cells	—	+ +	—	—

lary wall had led to total occlusion of the capillary lumen (Fig 9) Adhesions between the parietal and visceral epithelium were often seen and crescent formation was common (Fig 10) Periglomerular fibrosis was present in all cases

There was a strong interstitial fibrosis involving the cortical part of the kidney often extending even to the medulla (Fig 9) Infiltration of the interstitium with lymphocytes and with plasma cells was not as pronounced in these cases as in the former group On the other hand, tubular dilatations were more widespread and involved proximal as well as distal convoluted tubuli In many areas the dilated tubuli contained amorphous material, giving a thyroid like appearance

Electron Microscopic Findings

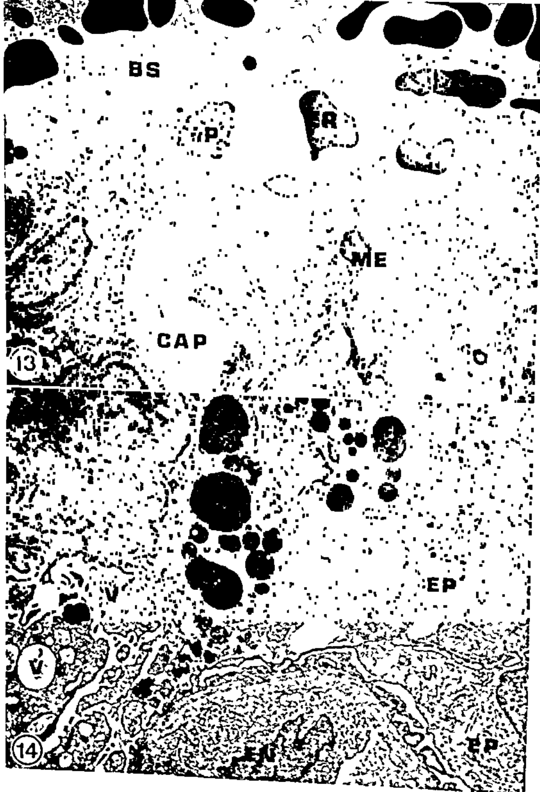
No glomerular abnormalities were recognized in the young control dogs The capillaries were widely open, the GBM was of even thickness, and no apparent lesions were observed in the epithelial, endothelial or in the mesangial cells (Fig 13) On the contrary, different forms of electron microscopic lesions were observed in the nephritic dogs as well as in the older control dogs, these lesions will be described in detail in the following The occurrence of different lesions is shown in Table 1

Epithelial cell Degenerative changes in the cytoplasm of the epithelial cells were seen only in nephritic dogs These changes included mitochondrial lesions, such as dispersion of the internal laminar structure of the mitochondria or occasional vacuolization, widening of the endoplastic reticular sacs and occurrence of different types of vacuoles in the cytoplasm (Fig 14) The latter were often

Occasionally desquamated epithelial cells

Fig 13 Electron micrograph of the kidney of a young control dog showing widely open *capillaries* (CAP) with some erythrocytes (ER) inside the capillary lumen and also in Bowmans Space (BS) EP – epithelial cell MT = mesangial cell EN – endothelial cell 3 000 ×

Fig 14 Electron micrograph of a nephritic dog with moder
showin.
(EP)
and the cytoplasm of that cell is amorphous Empty vacuoles (V) as well as vacuoles filled with coarse granular material (V) are present ML = mesangial cell EN endothelial cell 12 000 ×


BS
P
R
ME
CAP
13
V
V
EP
14
EN
EP

were seen the cytoplasm in these was composed of granular cell debris, remnants of cytoplasmic organelles being buried in this material (Fig 14)

As a rule electron dense deposits of finely granular character were observed in the epithelial cell cytoplasm of the nephritic kidneys, but such deposits were also seen occasionally in the older control dogs (Fig 15) No membrane separating these deposits from the remaining cytoplasm was observed

Fusion of epithelial foot processes was seen only in areas where the GBM was extensively thickened

Glomerular basement membranes The GBM of the young control dogs were of uniform structure varying from 2000 A to 3000 A in thickness In nephritic dogs, and also to some extent in the older control dogs, thickening of the GBM had occurred, the thickness varying between 6000 A and 10 000 A in these dogs This thickening was mainly due to the lamination of the lamina densa and splitting of this into two or more layers (Fig 16) If splitting was extensive, the GBM had a lattice like appearance (Fig 17) The lamina rara interna varied in thickness, whereas lamina rara externa usually had not widened Electron transparent areas, similar to the *laminae rarae*, were seen between the leaves of the split lamina densa The thickening of the GBM was irregular with accentuations either on the epithelial side (Figs 16, 17 and 19) or on the endothelial side (Fig 18)

Electron dense deposits of round or irregular form were seen as a rule in the GBM in the nephritic dogs, seldom in the older control dogs, and never in the young control dogs The deposits were composed of fine granular material which never had the fibrillar pattern characteristic of amyloid The localization of the deposits within GBM was either subepithelial (Fig 17) or subendothelial (Fig 18), depending upon whether the accentuation of the thickening of the GBM was on the epithelial or the endothelial side

Vacuoles with an electron dense rim were seen to some extent inside the GBM in the older control dogs and as a rule in the nephritic dogs (Fig 19) The vacuoles were either empty or they contained homogeneous material, evidently representing intramural lipid droplets

Endothelial cells Proliferation of endothelial cells were seen to some extent in the nephritic dogs but not in the control dogs Degenerative changes were seen in all nephritic dogs, in most cases there was a swelling of the endothelial cytoplasm resulting occasionally in a narrowing of the glomerular capillary lumen In addition, varying degrees of vacuolization occurred in the endothelial cytoplasm

Mesangial cells Mesangial proliferation of mild degree was seen only in the dogs with *moderate* light microscopic glomerular lesions In addition, basement membrane like *material* was seen in abundance in some dogs in this group

Myxovirus like structures were seen in the endothelium of some of the dogs, their morphology and distribution will be described in a separate report (12)

Immunofluorescence studies Staining of the cryostat sections of the kidneys with fluoresceinated antiserum to canine IgG was performed in all cases Staining along the capillary walls and of the mesangial stalk was seen in all nephritic dogs and to some degree in three of the older control dogs The characteristic pattern of the staining has been reported previously (11) an example is given in Fig 20

Correlation of BUN levels to the renal changes BUN was higher in the group of dogs with moderate glomerular changes (av-

Fig 15 Electron micrograph of an old control dog showing thickening and mottling of the glomerular basement membrane (BM) An electron dense deposit (D) of finely granular character is seen in the epithelial cell (EP) 15 000 ×

Fig 16 Electron micrograph of an old control dog showing thickening of the glomerular basement membrane (BM) due to splitting of the lamina densa There are accentuations on the epithelial side of the membrane CAP — capillary lumen, EP epithelial cell 25 000 ×

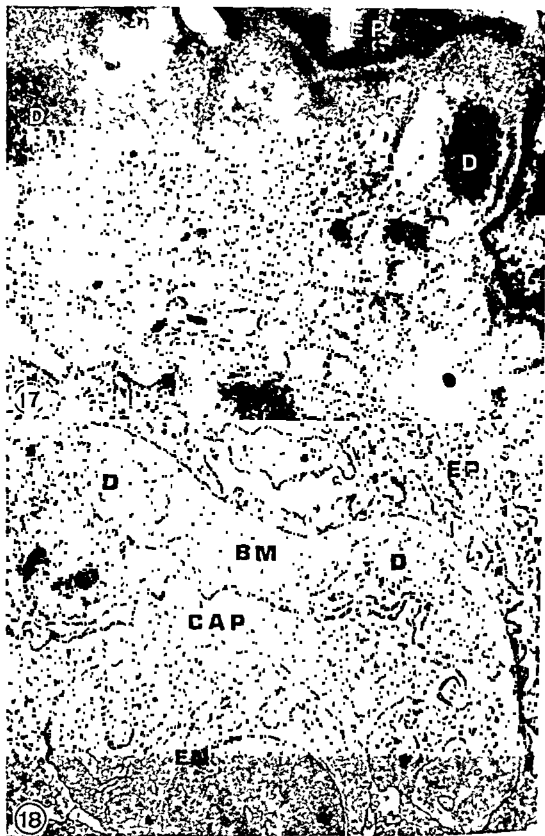

EP
D
D
17
EP
D
BM
D
CAP
EN
18

Fig 19 Electron micrograph of a nephritic dog with mild glomerular changes showing accumulation of vacuoles (V), evidently representing lipid droplets inside the basement membrane (BM) EP = epithelial cell CAP = capillary lumen 30 000 ×

Fig 17 Electron micrograph of a nephritic dog with moderate glomerular changes. The basement membrane is thickened due to extensive splitting of lamina densa into several leaves Fine fibrillar material is seen in between the separated leaves of lamina densa and two electron dense deposits (D) are located near the epithelial side of the basement membrane. EN = endothelial cell, EP = epithelial cell 60 000 ×

Fig 18 Electron micrograph of a nephritic dog with moderate glomerular changes The glomerular basement membrane (BM) is thickened due to the splitting of the lamina densa. Fine granular material as well as several electron dense deposits (D) are located on the endothelial side of the membrane. CAP = capillary lumen EN = endothelial cell FP = endothelial cell 20,000 ×



Fig 20 Glomerulus from a nephritic dog with mild glomerular changes stained with fluoresceinated antiserum to canine IgG showing the deposition of IgG type antibodies along the capillary walls and in the mesangium The staining is partly linear, partly granular 400 ×

erage 40 mg/100 ml, range 26 55) than in dogs with mild glomerular changes (average 22 mg/100 ml, range 15 32) In the cases of severe glomerular changes, BUN was estimated only in one case and found to be 65 mg/100 ml

DISCUSSION

It is well known that glomerular changes occur in connection with canine interstitial nephritis (CIN) no matter whether the disease is caused by Leptospirae or it is of unknown aetiology (1, 12) The significance of these changes is however, poorly understood and the pathogenetic mechanism leading to the glomerular changes has not been characterized Immunological injury of unknown character has been suggested to be cause of the lesion, although vascular changes with concomitant hypertension has also been considered (1) In the previous part of our work (11) we demonstrated the presence of precipitated IgG as well as complement component C3 in the capillary wall and in the mesangium of the affected glomeruli in this disease Elution studies revealed that antibodies eluted from the affected glomeruli did not react with any normal canine tissue but did react with diseased glomeruli indicating the presence of antibodies and of unknown antigen in the glomeruli at the same time The disease therefore seemed to belong to the group of diseases occurring spontaneously or experimentally in man and in some laboratory animals where the injury to the glomerulus might be caused by antigen antibody complexes In many of these diseases as e g in systemic lupus erythematosus (SLE) viral antigens have been suspected to be a part of the immune complexes while in others such as the kidney disease occurring in mice infected with lymphocytic choriomeningitis virus (LMC) (10) the viral aetiology of the disease has been proven

The present study confirms the findings obtained in previous light microscopic works on the renal histology in CIN according to which the regular occurrence of glomerular changes in this disease was demonstrated Furthermore, our findings seem to indicate that the progression of the glomerular lesions is closely associated with the process taking place in the renal interstitium Dogs with mild glomerular lesions had only slight interstitial inflammatory cell infiltration in the kidneys and no fibrosis whereas a pronounced amount of lymphocytes and plasma cells was present in the renal interstitium in cases where the glomerular changes were rated as moderate In kidneys presenting advanced signs of chronic glomerulonephritis the tubuli were extensively injured and there was a marked fibrosis Thus the progression of the disease seems to involve the glomerular component of the affected kidneys and at the same time the interstitium and tubuli

The light microscopic appearance of the moderately affected glomeruli resembles the membranoproliferative glomerulonephritis in general particularly the lesions seen in SLE (7 14) The thickening of the capillary walls was mainly due to accumulation of PAS positive fibrinoid material along them and wire loop lesions were occasionally seen However the proliferative component of the process was rather faint In electron microscopic study alterations of the basement membrane similar to those seen in SLE (6) in man were observed in the affected glomeruli of the dogs In general the thickened glomerular basement membranes contained deposits which were located on the subendothelial side of the membrane Occasional subepithelial deposits were seen but they may occur also in SLE although infrequently (5)

Glomerular lesions similar to those seen in the present material have also been noted in some kidney diseases in animal and assumed to be caused by the action of immune complexes The light microscopic patterns of glomerular lesions to occur in the spontaneous renal disease in NZB/NZW mice is similar to human SLE in many respects (4), electron microscopy has revealed the presence of subendothelial deposits in this condition (3 4) The lesions found in mice infected with LCM virus (10) as well as in minks affected

by Aleutian disease are also similar to those seen in our material The deposits on the epithelial side of the GBM seen in our cases, however, resemble rather the lesions found in nephrotoxic nephritis, as shown for example in experimental work with monkeys (2)

Although glomerular changes in CIN might occur secondarily to the process of interstitial inflammation and injury, the glomerulus itself may also be the primary site of the disease In the cases of mild glomerular lesions, the interstitial process was also mild and glomerular lesions were seen also in some of the older control dogs Some of these lesions, such as splitting of the GBM and deposition of lipid vacuoles inside the membrane, are probably related to the general ageing process However, more specific changes, such as epithelial and intramembranous electron dense deposits as well as deposition of immunoglobulin IgG in some parts of the capillary wall, were also observed This finding would indicate that a relatively common process leads to mild glomerular lesions in most of the ageing dogs and that the lesion in some of them progresses to overt disease accompanied by interstitial changes Our finding of myxovirus-like particles in the endothelial cytoplasm in some of the cases of CIN as well as in the older control dogs could indicate that this process may be related to an altered immunological response to some common canine virus, most probably to canine distemper virus This will be presented in detail in a subsequent paper The fact that CIN characteristically affects older dogs may indicate that this altered immunological response is in fact due to the fading of the immunity to this virus normally occurring in vaccinated young animals and could thus be attributed to the process of ageing (8)

This study was supported by grants from the *State Medical Research Council of Finland, Sigrid Juselius Foundation* and in part by the Grant HE 09011-07, *National Heart and Lung Institute, National Institutes of Health*

REFERENCES

1 *Anderson, Lindsay J* The glomeruli in canine interstitial nephritis J Path Bact *95* 59–65, 1968

2 *Battifora, H A & Markowitz, A S* Nephrotoxic nephritis in monkeys Sequential light, immuno-fluorescence and electron microscopic studies. Amer J Path *55* 267 281, 1969

3 *Channing, A A, Kasuga, T, Horowitz, R E, Dubois, L E & Demopoulos, H B* An ultra structural study of spontaneous lupus nephritis in NZB/NZW mouse Amer J Path *47* 677–694, 1965

4 *Camenford, F R, Cohen, A S & Desai, R G* The evolution of the glomerular lesions in NZB mice A light and electron microscopic study Lab Invest *19* 643–651, 1968

5 *Ehrenreich T & Churg, J* Pathology of membranous nephropathy In Pathology Annual Ed Sheldon C Sommers, Butterworths, London and Appleton Century Grofts, New York Vol 3, 145–186, 1968

6 *Faith, G C & Trump, B F* The glomerular capillary wall in human kidney disease Acute glomerulonephritis, systemic lupus erythematosus and preeclampsia-eclampsia Comparative electron microscopic observations and a review Lab Invest *15* 11, 1962–1719, 1966

7 *Grisman, E, Churg, J, Mautner, W & Suzuki, Y* Pathology of lupus nephritis J Mount Sinai Hosp, N Y *30* 117, 1963

8 *Guttman, P H* Renal Pathology In *The Beagle* as an experimental dog Ed Allen C Andersen The Iowa State University Press, Ames, Iowa Pages 546–558, 1970

9 *Henson, J B, Gorham, J R & Tanaka, Y* Renal glomerular ultrastructure in mink affected by Aleutian disease Lab Invest *17* 123 139, 1967

10 *Kajima, M & Pollard, M* Ultrastructural pathology of glomerular lesions in gnotobiotic mice with congenital lymphocytic choriomeningitis (LCM) virus infection Amer J Path *61* 2 117 140, 1970

11 *Krohn, K, Mero, M, Oksanen, A & Sandholm, M* Immunologic observation in canine interstitial nephritis Amer J Path *65* *1* 157–172, 1971

12 *Krohn, K & Sandholm, M* Myxovirus like structures in the glomerular endothelial cytoplasm in canine nephritis Submitted

13 *McIntyre, W I M & Montgomery, G L* Renal lesions in Leptospira Canicola infection in dogs J Path Bact *64* 145–160, 1952

14 *Muehrke, R C, Kark, R M, Pirani, C L & Pollak, V E* Lupus nephritis A clinical and pathologic study based on renal biopsies Medicine *36* 49–67, 1957

Acta path microbiol scand Section A *81*, 474–482, 1973

IMMUNOFLUORESCENT DEMONSTRATION OF BACTERIAL ANTIGEN IN EXPERIMENTAL PYELONEPHRITIS WITH ANTISERUM AGAINST COMMON ENTEROBACTERIAL ANTIGEN

OLE FROKJÆR THOMSEN and TAGE HJORT

University Institute of Pathology, Kommunehospitalet, and
Institute of Medical Microbiology, University of Århus, Denmark

Various strains of *E coli* were used to produce experimental haematogenous pyelonephritis in rats. The persistence in the renal tissue of the common enterobacterial antigen (CA) shared by these micro-organisms was then studied by means of indirect immunofluorescent technique with rabbit antiserum against CA In fresh infections, CA could regularly be demonstrated in and around abscesses in macrophages in dilated tubules and in pelvic exudate However, as early as 2 weeks in the course of the infection, CA was hardly detectable in macrophages By means of specific antiserum against one of the strains (*E coli* 04), it could, however, be shown that other antigens from this strain were still present in the macrophages in animals infected with this strain In renal tissue with chronic inflammatory changes examined more than 4 weeks after the bacterial injection, bacterial antigen could no longer be detected with antiserum against CA, whereas antiserum against *E coli* 04 still revealed positive reactions on renal tissue from the rats infected with this strain

A common enterobacterial antigen (CA) shared by nearly all strains of *E coli* and most other species belonging to Enterobacteriaceae was described by *Kunin* (5) in 1963 A strong antiserum against CA can be obtained by immunizing rabbits with *E coli* 014

Aoki et al (1) suggested that immunofluorescent tests with antibodies against CA might be an easy and useful way of demonstrating bacterial antigen in renal tissue, instead of working with many different specific antisera against different strains of *E coli* and other Enterobacteriaceae They therefore studied such a test system on bacteria *in vitro* (2) in experimental pyelonephritis (1) and in human chronic pyelonephritis (3) In the last mentioned investigation CA could be demonstrated not only in renal tissue from pyelonephritic patients with enterobacteria in the urine, but also in some cases of chronic pyelonephritis without demonstrable bacteria In particular, the positive results from patients without viable bacteria in the urine and

We have tried to take advantage of this technique in the study of renal tissue from patients with chronic pyelonephritis without

Received 4 1 73 Accepted 25 1 73

Requests for reprints should be addressed to *O Frøkjær Thomsen*, University Institute of Pathology, Kommunehospitalet, 8000 Århus C, Denmark

demonstrable bacteria However, the fluorescent tests were negative even though the test system worked well on smears of various enterobacteria grown *in vitro* The reason *might* be either that the antigen had never been present in the renal tissue, or that it had been eliminated from it

The latter possibility gave rise to the question For how long a period after renal infection with enterobacteria can CA be detected in renal tissue? As the literature gives only sparse information on that problem, we tried to elucidate it further in studies of experimental pyelonephritis in rats At the same time the persistence of CA in renal tissue was compared with that of other antigens from one of the *E coli* strains used in the experiments

MATERIAL AND METHODS

Experimental procedure Experimental haematogenous pyelonephritis was produced in white rats (Wi

[illegible]

against the dorsolateral wall musculature A polyethylene suture was passed around the ureter and both ends passed through the fascia and skin, after which the loose ends were tied over a piece of rubber tube Then 1 ml of a bacterial suspension containing approx. 1×10^8 viable organisms per ml was injected into a tail vein The ureter ligature was removed after 20 22 hours

Bacteria The injected bacterial strains were (1) *E coli* 014 and nine other strains of *E coli* representing the following 0-antigens 02, 04, 06, 07, 08 09, 018, 022, 075, (2) one strain of *Proteus mirabilis*, and one strain of *Streptococcus faecalis* (The strains of *E coli* which were the antigenic test strains for the respective *Escherichia coli* 0-groups were kindly supplied by Dr F Ørskov, International Escherichia Centre Statens Serum Institut Copenhagen) One colony from a blood *agar plate was grown in a brain heart broth at* 37° C for 24 hours After centrifugation, the bacteria were suspended in sterile saline to a concentration of 1×10^9 viable organisms. This number was checked by counting the number of colonies obtained on blood agar plates after spreading 0 01 ml of increasing dilutions of the stock suspension

Sampling of kidney tissue After the injection of bacteria the animals were killed at intervals by heart puncture exsanguination under intraperitoneal pentobarbiturate anaesthesia About one third of the infected kidney was removed aseptically for bacterial culture The remaining kidney tissue was quick frozen in isopentane precooled to −70° C The blocks of frozen tissue were stored at that temperature until required They were sectioned in a cryostat at −20° C Consecutive sections, 6 μ in thickness, were placed on cleaned slides, fixed in acetone for 10 minutes, and then dried at room temperature for 30 minutes before staining In most cases part of the kidney tissue was also prepared by the method of *Sainte Marie* (9) with 95 per cent *ethyl alcohol at 4° C as the first fixative, and paraf*fin as the embedding medium The paraffin blocks *were stored at 4° C until required* Sections, 6 μ in thickness, were cut at room temperature before staining

Sections of tissue prepared by these methods were used partly for light microscopic examination after staining with haematoxylin eosin and periodic acid Schiff (PAS), partly for immunofluorescence studies, for which both methods proved to be suitable *The Sainte Marie method generally yielded the* best morphological presentation of the tissue

Immi
stained
techniq
rabbit antiserum against bacterial antigen—either *anti CA or anti E coli 04—and the second layer a*

[illegible]

werke)

The proper dilutions of the various antisera and conjugates were determined by titration on smears of bacteria. Phosphate buffered saline (PBS) was used as diluent Before adding the antisera, slides were washed in PBS (pH 7 1) for 3 × 5 minutes Then the first layer was applied for 30 minutes. After washing in PBS for 3 × 5 minutes, the second layer was applied for 30 minutes The slides were then washed in PBS for 3 × 5 minutes, dried and mounted in Fluomount

Antisera against *E coli* 014 (anti CA serum) and *against E coli 04 (anti 04 serum) were pro*duced in rabbits by biweekly intravenous injections of suspensions of *E coli* 014 and *E coli* 04, respectively, which had been killed by heating at 100° C for a few minutes (from about 4×10^7 micro-organisms in the first injection to about 4×10^8 in the last injection after 3 weeks) The antibody production was followed by titration of the sera in immunofluorescent tests on smears of the various strains of *E coli*, and by haemagglutination with cells coated with lipopolysaccharide extracts *from E coli* 014 *and the various other strains* mentioned above The coating procedure was carried out as described by *Kunin* (6) The haemagglutination tests were carried out in a microtitre system by mixing 0 025 ml of increasing dilutions of serum (1 2, 1 4 1 2048) with an equal

volume of a 1 per cent suspension of coated cells Both the anti CA serum and the anti 04 serum yielded titres ≧ 2048 with cells coated with the homologous antigen The anti CA serum also agglutinated cells coated with heterologous *E coli* antigens in fairly high titres (≧ 512), whereas the anti 04 serum gave only weak reactions

Microscopic examination The slides were examined under a Zeiss Universal Fluorescence Microscope with a Tiyoda condenser and an Osram HB 200 mercury lamp as the light source The primary filter was an interference filter specially adapted for fluorescein isothiocyanate (*Rygaard & Olsen*, 8) As the secondary filter type No 50 was used In photography the Kodak High Speed Ecta chromofilm was used During the microscopic examination we attempted as far as possible, to observe and photograph the same fields as they occurred in the consecutive sections This facilitated comparative studies of the specifically stained section and the control section

Specificity controls With the indirect immunofluorescence method the following control sections were used

1 Sections on which the first layer applied was a normal rabbit serum instead of the specific rabbit antiserum As normal rabbit serum appeared to contain small amounts of antibody against CA it was absorbed with *E coli* 014 before use in order to remove antibodies against this strain The second layer applied was as described above

2 Sections on which only the FITC labelled second layer was applied

3 Unstained sections and sections which had only been washed with PBS

4 As a further control the stainings were carried out on normal non infected rat kidneys

Fluorescent structures were compared with exactly the same structures in the consecutive control sections Only if fluorescence in these was totally absent or at least definitely weaker than that of the sections stained with antiserum it was considered to be specific

RESULTS

Gross Kidney Lesions

Only kidneys showing macroscopic evidence of acute or chronic pyelonephritis were used for further studies

Among 48 left sided kidneys removed 3–19 days after the injection of bacteria 23 showed changes of acute pyelonephritis Twenty five kidneys were excluded viz 11 with severe pyonephrosis, presumably because the passage through the ureter had never been re established after the removal of the ligature, and 14 without macroscopic lesions at all

Among 42 kidneys removed after more than 4 weeks, only six were contracted indicating chronic pyelonephritis, with no evidence of pyonephrosis The remaining 36 were excluded, viz 16 with pyonephrosis and 20 without gross lesions

Some bacterial strains produced renal infection fairly readily, while others did so only to a slight extent Thus, injection of *E coli* 014 resulted in infection, interpreted as acute pyelonephritis, only in one rat kidney out of 14

In none of the animals gross changes were seen in the right kidney

Acute Pyelonephritis

The 23 kidneys with gross lesions indicating acute pyelonephritis, with no or only slight pyonephrosis, showed enlargement from slight up to twice their normal size Abscesses ranging in size from 1 mm up to large confluent purulent lesions were seen sometimes with a flattened maplike appearance on the surface of the kidney Such abscesses occurred both in the medulla and especially, in the cortex although without any definite pattern of localization Bacterial cultures were positive in all cases

a *Light microscopy* Examination of haematoxylin eosin and PAS stained sections revealed severe acute inflammation In the cortex and medulla, large and small infiltrations of polymorphonuclear leucocytes were seen and—at the later stages of the infection—mononuclear cells as well Both rounded abscedent foci and streaky radial collections of inflammatory cells were observed, spreading through the cortex and medulla down to the tip of the papilla often being of a wedge-shaped appearance with the broad end in the cortical surface In inflamed areas both atrophic and markedly dilated tubules and collecting tubules were seen containing a material of polymorphonuclears and necrotic debris The epithelial cells were flattened The glomeruli were intact and vascular changes were absent

The pelvis and calyces were dilated to a varying degree, often only slightly, and beneath the epithelium usually dense infiltrations, mainly of lymphocytes were seen The epithelium itself was rarely the site of inflammatory changes The lumen of the pelvis and calyces often contained exudates filled with leucocytes and cell debris

No differences were observed in kidneys infected with different bacterial strains

b *Immunofluorescence with anti C4 serum*

No evidence of specific fluorescence was seen in non infected rat kidneys or in the control sections from infected kidneys (Fig 1)

With the anti CA serum, bacterial antigen could be demonstrated, not only in the kidney infected with *E coli* 014, but also in the kidney tissue infected with the other nine strains of *E coli* (Table 1) The bacterial antigen appeared partly as recognizable organisms with a brilliant apple green fluorescence, partly as amorphous irregular clumps of fluorescent material

Recognizable bacteria were mainly localized in abscedent areas dilated tubules and in the pelvic exudate (Fig 6) Fluorescent material representing amorphous bacterial antigen could be demonstrated (1) in dilated tubules in the cortex and medulla (Fig 2), apparently mostly phagocytized by polymorphonuclears, which were here present in large numbers, (2) interstitially in cortical and medullary abscesses, and in the areas surrounding abscesses, apparently here phagocytized by macrophages, (3) often in the pelvic exudate, phagocytized by polymorphonuclears and (4) rarely in the pelvic epithelium

Bacterial antigen was not seen in the glomeruli or in relation to blood vessels There seemed to be certain differences in the intensity and extension of specific fluorescence produced by the various bacterial strains However, this is hardly possible to investigate in tissue sections

Kidney tissue infected with *P mirabilis*, which contains CA, revealed an appreciably weaker fluorescence than the kidneys infected with the coli strains

Kidneys infected with *Str faecalis*, which does not contain CA, did not exhibit specific fluorescence

c *Immunofluorescence with anti 04 serum*

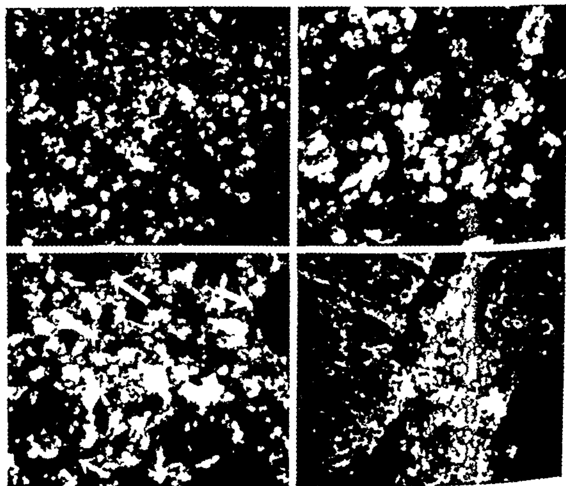
Applied to kidney tissue with acute pyelonephritis, anti 04 serum was able to yield specific fluorescence, indicating the presence of both amorphous bacterial antigen, and whole organisms in the kidneys infected with *E coli* 04 In contrast, with this antiserum it was generally not possible to demonstrate bacterial antigen in kidneys which were infected with the other coli strains. In a few cases, a very weak fluorescence was seen in the abscesses and dilated tubules, suggesting that this antiserum might have a low content of antibody against CA, this was confirmed in haemagglutination experiments

d *Comparison between the two antisera applied to kidney tissue infected with* E coli *04*

In the kidney that was taken out 5 days after the initiation of the infection both antisera yielded strong specific fluorescence, indicating the precence of both whole organisms and amorphous bacterial antigen in the aforementioned localizations

In the kidney that was removed after 6 days a similar picture was seen when anti CA-serum was used However, when using anti-04 serum, some amorphous interstitial antigen, probably phagocytized by macrophages, was observed, this material revealed a comparitively weaker fluorescence in the anti CA-stain (Fig 3 and 4)

Examination of the kidney tissue removed after 14 days revealed conspicuous differences Both in abscedent foci and in dilated tubules, a stronger and more extensive fluorescence was seen with anti 04 serum than with anti CA-serum. In addition, with anti-04 serum, specifically fluorescent structures could be demonstrated, which, when anti CA-serum was used, did not yield fluorescence at all, or only to a very slight degree in some



localizations The fluorescent material was amorphous interstitial antigen, the greater part of which was present in cells that seemed to be macrophages These cells had a brightly fluorescent cytoplasm and were seen in very large numbers, especially in the cortical area

Chronic Pyelonephritis

The six rats in which chronic pyelonephritic lesions were observed after 31–76 days had been injected with *E coli* 04 (three) or *E coli* 09, 018 or 022 (one each)

These kidneys were all contracted below normal size, and with an uneven surface There were no purulent areas or dilatation of the pelvis

a *Light microscopy* Typical chronic inflammatory changes were seen Both cortical and medullary areas were reduced in size Collections of inflammatory cells extended through the tissue from the pelvis to the cortical surface The cells were predominantly mononuclears, with the appearance of small lymphocytes, occasionally, small groups of polymorphonuclears were also present In inflamed areas, fibrosis and subsequent contraction of the cortical surface were observed

---

Fig 1 Dilated tubule with weakly autofluorescent material in section stained with a normal rabbit serum. 5th day of infection × 300

Fig 2 Same area as in Fig 1 in a consecutive section stained with anti CA serum The tubule contains amorphous clumps of bacterial antigen with brilliant specific fluorescence × 300

Fig 3 Cortical area showing macrophages with moderately fluorescent cytoplasm in section stained with anti CA-serum 6th day of infection × 300

Fig 4 Same area as in Fig 3 in another section stained with anti-04-serum A stronger cytoplasmic fluorescence in the macrophages is observed. × 300

Fig 5 Cortical area in a contracted kidney in section stained with anti 04-serum Specifically fluorescent interstitial clumps and macrophages among the closely packed glomeruli (arrows) 32nd day of infection × 300

Fig 6 Pelvic exudate with fluorescent amorphous antigen and whole organisms in section stained with anti-CA serum 14th day of infection × 300

The tubules were here atrophic and partially compressed, or dilated The glomeruli were close to each other, but unchanged In some juxtaglomerular arterioles, PAS positive hyaline changes were seen The pelvis was not dilated Beneath the epithelium, collections of mononuclear cells were present *The epithelium itself was without inflammatory changes* There were no abscesses

b *Immunofluorescence with anti CA-serum*

None of the six kidneys showed evidence of specific fluorescence, indicating the presence of amorphous bacterial antigen when anti-CA serum was used However, whole organisms could be demonstrated in the pelvic exudate from one of the kidneys infected with *E coli* 04, and, in agreement with this, bacteria could be cultured from the renal specimen.

b *Immunofluorescence with anti 04 serum*

When using anti 04 serum, bacterial antigen could not be demonstrated in the three kidneys which had not been injected with *E coli* 04 In the kidneys that had received *E coli* 04, the anti 04 serum revealed specific fluorescence, indicating the presence of amorphous bacterial antigen in the parenchyma This was localized interstitially, mainly in macrophages, both in the medulla and cortex, and chiefly in the areas where the infiltration of inflammatory cells was most pronounced (Fig 5) In addition, fluorescent whole organisms were seen in the pelvic exudate from the kidney with positive bacterial culture

It should be added that much autofluorescent material was seen in the chronically inflamed kidneys, this appeared as yellowish clumps and granules in the inflamed areas However, specific greenish yellow fluorescence could be distinguished from the autofluorescence under the aforementioned optical conditions with the use of an interference filter adapted to fluorescein isothiocyanate, the specific greenish yellow colour was also absent in the control sections, in contrast to the autofluorescence which was of the same

TABLE 1 *Results of Bacterial Cultures and Immunofluorescent Examination of Rat Kidneys*

Bacteria	Kidney tissue with acute pyelonephritis (3–19 days after infection)					Kidney tissue with chronic pyelonephritis (31–76 days after infection)				
	Number of kidneys	Number of days after infection	Bacterial culture	Bacterial antigen demonstr with anti CA	Bacterial antigen demonstr with anti 04	Number of kidneys	Number of days after infection	Bacterial culture	Bacterial antigen demonstr with anti CA	Bacterial antigen demonstr with anti 04
E coli 02	1	6	+	+	—					
E coli 04	3	5	+	+	+	3	31	—	—	+
		6	+	+	+		32	+	—*	+
		14	+	+	+		32	—	—	+
E coli 06	2	5	+	+	(+)					
		5	+	+	(+)					
E coli 07	3	3	+	+	(+)					
		6	+	+	(+)					
		19	+	+	(+)					
E coli 08	1	7	+	+	—					
E coli 09	1	11	+	+	—	1	74	+	—	—
E coli 014	1	19	+	+	—					
E coli 018	1	10	+	+	—	1	40	—	—	—
E coli 022	2	5	+	+	—	1	76	—	—	—
		14	+	+						
E coli 075	1	6	+	+	—					
P mirabilis	2	5	+	(+)	—					
		5	+	(+)	—					
Str faecalis	5	14	+	—	—					
		15	+	—	—					
		15	+	—	—					
		18	+	—	—					
		18	+	—	—					

* Thus whole bacteria in the pelvic lumen

appearance in all sections, including the unstained ones

DISCUSSION

In kidney tissue with acute pyelonephritis, bacterial antigen from various *E coli* strains, both in the form of whole organisms and amorphous material, could be detected by means of the antiserum against CA This was expected from the results of other investigations (1, 2 3) and our own haemagglutination and immunofluorescence tests on bacteria *in vitro*

The intensity of the fluorescence seemed to show some variation in the reactions between anti CA serum and the various strains of *E coli* However, it will hardly be possible to make a quantitative estimation of this phenomenon in kidney tissue

By using antiserum against one of the infecting strains, the homologous organism (*E coli* 04) could, as might have been expected, be demonstrated very clearly Applied to most of the heterologous bacteria no reaction was seen, but in a few cases a weak reaction occurred, due to a low content of antibody against CA in the anti 04-serum

In the examination of the three kidneys infected with *E coli* 04, presenting the picture of acute pyelonephritis, a more intense fluorescence was noted when the specific anti serum was used instead of the anti CA serum This is natural, since—according to the procedure used for its production—the specific antiserum should contain antibodies against several antigenic determinants in the bacteria

A more remarkable observation was that the specific antiserum in two of the three kidneys in addition to revealing bacterial antigen in amounts and localizations which could also be demonstrated with anti CA serum, was able to localize amorphous bacterial antigen in numerous macrophages and interstitial clumps, with anti CA serum no, or in a few cases only a very weak, fluorescence was observed in these structures

This points in the direction of lack of parallelism of the occurrence in the tissue of CA and specific antigens, which also seemed to be the case in kidney tissue with older inflammatory changes Thus, in none of the six kidneys presenting the gross and microscopic lesions of chronic pyelonephritis, amorphous bacterial antigen could be demonstrated in the parenchyma by means of anti CA serum, However, when the specific anti 04 serum was used, clumps of bacterial antigen located in numerous macrophages could be demonstrated very clearly in the three of the kidneys that were infected with *E coli* 04

As to the persistence of bacterial antigen in the kidney tissue with experimental pyelonephritis, it should be noted that *Sanford et al* (10), in haematogenous pyelonephritis in rats, demonstrated amorphous bacterial antigen after 10 weeks, and so did *Cotran* (4) in retrograde pyelonephritis in rats after 20 weeks, in both immunofluorescence investigations specific antiserum was used

The explanation of the present observations might be that in the course of the inflammation CA is removed, altered or neutralized by antibody in such a way that it becomes unable to react with the antiserum against it Thus, in our system, CA was less persistent in the tissue than the specific antigens This is in disagreement with the results of *Aoki et al* (1), who demonstrated that the localization and persistence of the common antigen closely paralleled that of specific antigen However, these investigators used mice and injected heat killed bacteria percutaneously direct into the kidneys in amounts comparable with that present in experimental pyelonephritis Under these circumstances, CA was demonstrable for a period of 6 weeks after injection, and specific antigens could be detected for 7 weeks

In another connection, *Miller et al* (7) mentioned that they had detected common antigen and specific antigen about 6 months after intrarenal injection of *E coli* 075 in two rabbits

In the light of the present investigation, the *immunofluorescence technique* using antiserum against CA may be regarded as a suitable method for the demonstration of bacte-

rial antigen in strains of *E coli* in recent kidney infections which are characterized by acute inflammation and production of pus

In deciding whether the method is applicable in the study of renal tissue with chronic inflammatory changes, it should be borne in mind that we managed to produce true chronic pyelonephritis only in six out of 42 cases However, in view of the completely negative reaction in all six cases, we regard it as questionable whether the method is suitable when the infection has reached a subchronic or chronic stage As mentioned before, the fact that the same strains of *E coli* were clearly demonstrable in acute pyelonephritis seems to point in the direction that CA disappears or is altered after a fairly short period, and cannot be disclosed in the tissue at a time when the specific antigen is still demonstrable

Although caution should be exercised in drawing conclusions from animal experiments to human conditions, these experiences from rat infections make our negative results of immunofluorescent studies of human kidney tissue with chronic pyelonephritis more conceivable

REFERENCES

1 *Aoki, S , Merkel, M , Aoki, M* & *McCabe, W R* Immunofluorescent localization of bacterial antigen in pyelonephritis I The use of anti sera against the common enterobacterial antigen in experimental renal lesions J Lab clin. Med *70* 204–212, 1967

2 *Aoki, S , Merkel, M* & *McCabe, W R* Immunofluorescent demonstration of the common enterobacterial antigen Proc Soc. exp Biol Med *121* 230–234, 1966

3 *Aoki, S , Imamura, S , Aoki, M* & *McCabe, W R* "Abacterial" and bacterial pyelonephritis Immunofluorescent localization of bacterial antigen New Engl J Med *281* 1375–1382, 1969

4 *Cotran, R S* Retrograde proteus pyelonephritis in rats localization of antigen and antibody in treated sterile pyelonephritic kidneys. J exp Med *117* 813–822, 1963

5 *Kunin, C M* Separation characterization and biological significance of a common antigen in Enterobacteriaceae J exp Med *118* 565–586, 1963

6 *Kunin, C M* Antibody distribution against nonenteropathic *E coli* Arch intern Med. *110* 676–687, 1962

7

Br J exp Path *52* 678–683, 1971

8 *Rygaard, J* & *Olsen, W* Interference filters for improved immunofluorescence microscopy Acta path microbiol scand *76* 146–148, 1969

9 *Sainte Marie, G* A paraffin embedding technique for studies employing immunofluorescence J Histochem Cytochem. *10* 250–256, 1962

10 *Sanford, J P , Hunter, B W* & *Donaldson P* Localization and fate of Escherichia coli in hematogenous pyelonephritis J exp Med *116* 285–294 1962

Acta path microbiol scand Section A 81, 483–497, 1973

OBSERVATIONS ON THE SURFACE ARCHITECTURE OF HISTAMINE-INDUCED GASTRIC ULCERATIONS IN THE GUINEA PIG

STEEN SEIER POULSEN

Anatomy Department B (Head Professor E Landboe Christensen, M D),
University of Copenhagen Denmark

Gastric ulcerations were induced in adult male guinea pigs by daily injection of a slowly absorbed histamine preparation for up to 18 days So that the various stages of the ulceration process could be studied as thoroughly as possible, the ulcerogenic action was rendered as constant and gentle as possible Therefore the animals were not fasted before or during the experiment, and they were protected from the acute musculo-vascular effect of histamine by a low dose of promethazine prior to each injection of histamine In the course of the first five experimental days numerous superficial erosions appeared, but true ulcers which had penetrated the lamina muscularis mucosae had not formed until the 5th 10th day At the end of 10 days a number of the superficial erosions had regenerated After 18 days the tendency to new formation of erosions had decreased, and many of the superficial erosions had regenerated The specimens were studied in the stereomicroscope after periodic acid Schiff (PAS) staining of whole mounts and thereafter by conventional histological technique The surface morphology and histological appearance of the various stages of the ulceration process and of the regenerated ulcerations are described

In 1942 Hay et al (10) described a reliable method for inducing gastroduodenal ulcers in various experimental animals by means of slowly absorbed histamines Since then, histamine has been widely used in the study of experimental gastric and duodenal ulcerations (2, 7, 9, 19, 22, 23) The most commonly used animal has been the guinea pig, because unlike the rat it needs only comparatively low doses of histamine to develop ulcerations

Only a few authors have reported on the initial stages of the ulcerative process Watt (22), in 1959, described the histopathological mechanism whereby hyperacute gastric ulcerations arose. He used fasting guinea pigs which after one injection of histamine developed ulcerations in less than four hours He found that these ulcerations arose as focal, ischaemic necroses

The object of the present paper is to describe the morphological appearances and the morphological development of ulcers induced in a slower and gentler way Thus, the animals were not fasted before or during the experiment, and by a small dose of promethazine they were protected from the acute musculo vascular effect of histamine

While previously the changes of the gastric mucosa have been followed either by direct inspection of the living mucosa in a stereo microscope or else by conventional histological technique, the stomachs of the present ex-

Received 6 xii 72 Accepted 16 i 73

rial antigen in strains of *E coli* in recent kidney infections which are characterized by acute inflammation and production of pus

In deciding whether the method is applicable in the study of renal tissue with chronic inflammatory changes, it should be borne in mind that we managed to produce true chronic pyelonephritis only in six out of 42 cases However, in view of the completely negative reaction in all six cases, we regard it as questionable whether the method is suitable when the infection has reached a subchronic or chronic stage As mentioned before, the fact that the same strains of *E coli* were clearly demonstrable in acute pyelonephritis seems to point in the direction that CA disappears or is altered after a fairly short period, and cannot be disclosed in the tissue at a time when the specific antigen is still demonstrable

Although caution should be exercised in drawing conclusions from animal experiments to human conditions, these experiences from rat infections make our negative results of immunofluorescent studies of human kidney tissue with chronic pyelonephritis more conceivable

REFERENCES

1 *Aoki S*, *Merkel, M*, *Aoki M* & *McCabe, W R* Immunofluorescent localization of bacterial antigen in pyelonephritis I The use of anti sera against the common enterobacterial antigen in experimental renal lesions J Lab clin.

2

enterobacterial antigen Proc Soc. exp Biol Med *121* 230–234, 1966

3 *Aoki, S*, *Imamura, S*, *Aoki, M* & *McCabe W R* 'Abacterial' and bacterial pyelonephritis. Immunofluorescent localization of bacterial antigen New Engl J Med *281* 1375–1382 1969

4

5

gen in Enterobacteriaceae J exp Med *118* 565–586, 1963

6 *Kunin, C M* Antibody distribution against nonenteropathic *E coli* Arch intern Med. *110* 676–687, 1962

7 *M*

Br J exp Path *52* 678–683 1971

8 *Rygaard, J* & *Olsen W* Interference filters for improved immunofluorescence microscopy Acta path microbiol scand *76* 146 148 1969

9 *Sainte Marie, G* A paraffin embedding technique for studies employing immunofluorescence J Histochem Cytochem *10* 250–256, 1962

10 *Sanford, J P*, *Hunter B W* & *Donaldson P* Localization and fate of Escherichia coli in hematogenous pyelonephritis J exp Med. *116* 285–294, 1962



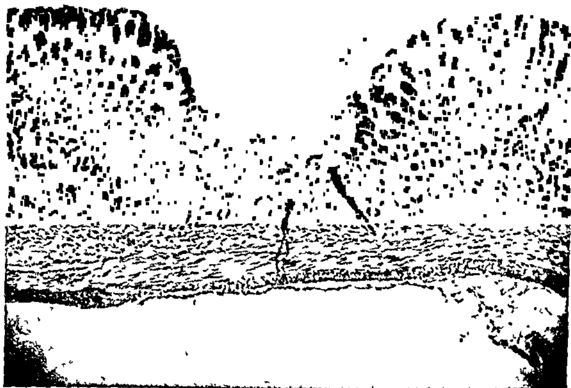


Fig 2 Histologic section of U shaped, superficial erosion in stage I PAS hematoxylin aurantia. × 75

examined in the stereomicroscope, standing out distinctly as pale, sharply defined structures on the deep purple background After stereomicroscopic inspection, and photographing in some cases, ordinary histological sections of the changed parts were prepared By comparing the surface appearance with the changes seen in the histological sections it was possible to set up three well defined morphological pictures which below will be designated stage I, II, and III

Stage I

Surface structure The changes were seen primarily in sulci between areae gastricae, appearing as isolated fissure-shaped erosions (Fig 1) or a network of erosions surrounding one or more areae gastricae (Fig 3) The sulci were widened at the site of the erosions whose bed showed a pale, homogeneous surface, forming sharp contrast to the normal, PAS-positive surface Whereas in regions with few, non-confluent erosions the areae gastricae appeared normal and were normally PAS positive, signs of varying degeneration were seen in areae gastricae which were completely surrounded by erosions In these sites there was reduced stainability and partially obliterated surface structure, where the foveolae (gastric pits) were smaller than normal, some of them completely obliterated

Histologically each erosion showed as a superficial, almost u-shaped defect extending

Fig 3 Network of stage I and II ulcerations PAS staining × 25 A Unaffected area gastrica sur-

trica


A
B
C

down through about two-thirds of the mucous membrane The glandular structure at the bed of the erosion was partially obliterated, and there was slight infiltration by polymorphonuclear leukocytes The lamina muscularis mucosae was not involved (Fig 2)

Stage II

Surface structure The isolated areae gastricae had been destroyed by necrosis and desquamated, leaving a wider erosion with a somewhat deeper-lying central area which was irregular and somewhat darker than the surrounding part of the erosion which corresponded morphologically to a stage I erosion *The central part* was delimited by a distinct border and might contain remnants of the *desquamated tissue* (Fig 3) *The ulceration* was in sharp contrast to the surrounding mucosa which remainded unaffected except for mild enlargement of the foveolae and an increased PAS positive reaction in the adjacent area Where many areae gastricae were involved in the process there might occur a major flat-bottomed erosion containing perhaps a few *islets of entirely or partly intact* areae gastricae

Histologically there was a wide, flat erosion whose bed in the marginal zone corresponded to the appearances of a stage I erosion whereas in the central part the lamina muscularis mucosae was covered only with a thin, necrotic layer infiltrated by granulocytes (Fig 4) There were signs of incipient degeneration in the lamina muscularis mucosae and violent oedema and granulocytic infiltration in the *submucosa Later, there might be necrosis of* the muscularis mucosae, thrombosis of the vessels in the submucosa, and oedematous splitting of the tunica muscularis

Stage III

Surface structure Now, the ulcerations were deeper, corresponding to the dark, central part observed in stage II (Fig 5) There might be a fully developed ulcer in which the

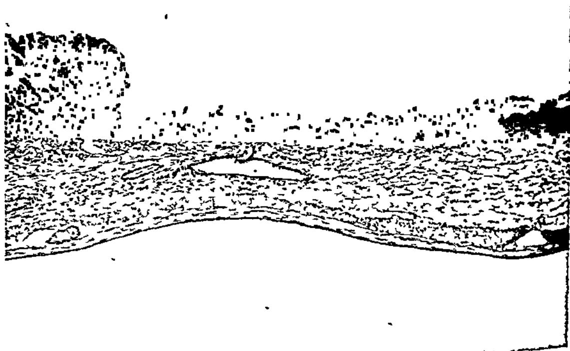


Fig 4 Histologic section of flat bottomed stage II ulceration Lamina muscularis mucosa unaffected oedema and slight inflammation in the submucosa Vessels uninvolved PAS hematoxylin aurantia × 50



Fig 5 Early stage III ulceration *Outlines* of the previous stage II ulceration still visible PAS staining × 25



Fig. 6 Full-blown stage III ulceration PAS staining × 25



Fig. 7. Histologic section of stage III ulceration PAS hematoxylin aurantia × 20

outlines were more rounded, the edges higher and undermined, and the areae gastricae as well as the interareolar grooves radiated towards the ulcer (Fig 6). Perforation might be present

Histologically the appearance was one of deep, penetrating ulceration with undermined edges The bed consisted most superficially of fibrin, leukocytes, and necrotic tissue Below the surface there was granulation tissue, and deepest down loose collagen connective tissue with ample capillary proliferation, especially in the subserous and peripheral parts of the ulceration (Fig 7)

Regeneration

Owing to the confluence of the ulcerations it was not possible to count them, so that the percentage distribution of ulcerations in the three stages cannot be stated, but at an estimate the ratio between ulcerations in the first two stages and those of stage III was about 20 1 Thus, only a relatively small proportion of the ulcerations were found to have reached stage III In most cases, the process of ulceration had stopped before that stage, and regeneration had set in

The regenerated ulcerations stood out distinctly as flat, slightly depressed, sharply defined islets in the normal mucosa (Fig. 1). Ulcerations which had just started regenerating were covered with an almost smooth, pale, but gradually increasing PAS-positive layer In this layer there formed some large, irregular foveolae, observed first peripherally in the ulceration (Fig 8) Gradually they got smaller, but whether eventually they assumed the appearance of the foveolae in the surrounding uninvolved tissue could [illegible] determined owing to the relatively [illegible] tion of the experiments

The histological appearance of [illegible] rated ulcerations corresponds [illegible] tion which *Williams* (21), in [illegible] of healing in experiment[illegible] ulcers In a healed ul[illegible] contained single, tu[illegible]

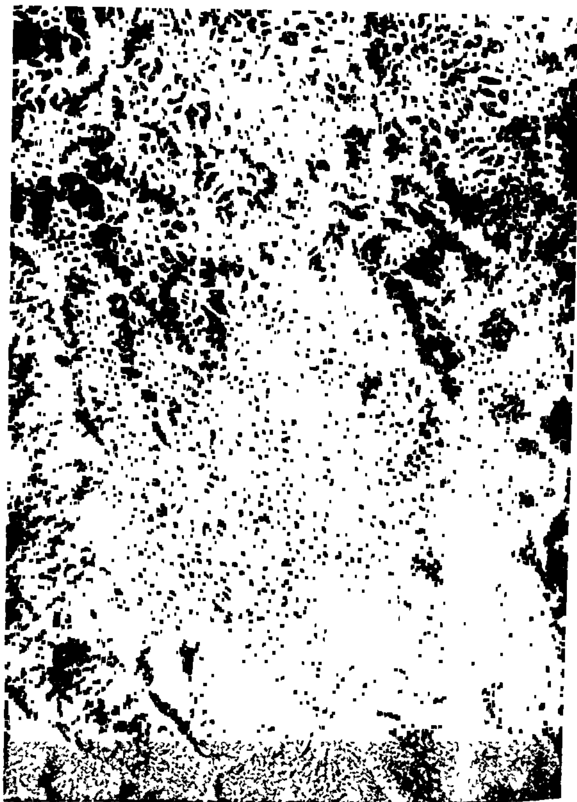


Fig 8 Regenerated ulceration Large, irregular gastric pits contrasting to the normal small gastric pits at the bottom of the picture PAS staining × 25

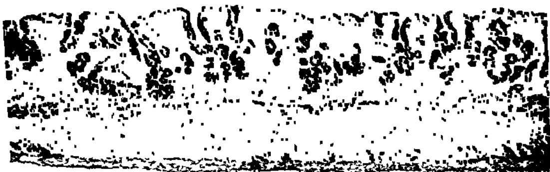


Fig 9 Histologic section of regenerated ulceration PAS-hematoxylin-aurantia × 50

glands debouching into deep, pylorus-like foveolae The muscularis mucosae was unaffected, but there might be connective tissue proliferation, oedema, and slight leukocytic infiltration in the submucosa There were no thrombosed vessels in the submucosa (Fig 9)

Course of Ulceration Process

There was a marked difference in the number of ulcerations in each stage between the various experimental groups

Within the group of guinea pigs killed after 5 days all the animals showed many stage I-II ulcerations, but only one had a small stage III ulcer and none showed ulcerations in a regenerative phase.

In the group killed after 10 12 days there were more ulcerations than in those of the previous group Six out of 9 guinea pigs had one or more stage III ulcers, including one which had perforated and showed the omentum as its bed Seven out of the 9 guinea pigs had ulcerations in the process of healing

In the guinea pigs killed after 18 days there were fewer active ulcerations than in the other two groups, but still there were many in stages I and II Three of the 8 guinea pigs had stage III ulcers The majority of the ulcerations were in the process of healing

Summing up, it may be said that stage I-II ulcerations were present in large numbers after only 5 days, whereas ulcers in stage III and in the process of healing were not observed until the group killed at 10 days In those killed after 18 days the number of new-formed ulcerations was decreasing

Out of the 6 controls one had two stellate ulcerations in the body of the stomach These ulcerations were small and superficial The other five controls had no ulcerations

DISCUSSION

The effects of histamine upon the stomach are well-known (3, 13, 18, 20). They comprise vigorous stimulation of the HCl-pepsin production, an increase in gastric peristalsis

or at a high histamine concentration, possibly tonic contraction of the gastric muscles and a vascular effect consisting in stasis, reduced flow, reduced O tension, and acidosis in the gastric mucosa

Which of the histamine effects is of most *importance in the development of histamine* induced gastric ulcers has been a matter of much discussion Presumably this varies with the experimental method used (7 10 12 14, 15 22 23)

Watt, in 1959 studied the course of the ulceration process by directly following it *in vivo* in guinea pigs *whose stomachs were* opened and distended under ether anaesthesia and inspected under the stereomicroscope He found the process to consist in four stages resulting in the formation of superficial ulcerations The entire process lasted for 1/2 3 hours (27)

Watt s experiments differ from the present ones both in method and in results Watt induced the ulcerations by an extreme acid action upon the mucosal surface It was obtained int al by fasting the animals before the injection of histamine so that during the experiment the mucosa totally lacked the acid pepsin diluting and neutralizing effect of the food as protection from the artificially increased acid production At the same time the fixation of the stomach prevents elimination of the acid by means of gastric peristalsis Therefore the ulcerations arise as acute focal necroses

Olovson (19) in 1950 was one of the first to supplement the ulcerogenic effect of histamine by 24 hours fasting before the experiment Since then nearly all studies have dealt with hyperacute ulcers arising in the course of less than 6 hours in fasting guinea pigs

In the present study it was endeavoured to make the process of ulceration slower and thus less acute in order better to distinguish and study its various stages This was attained by using an ulcerogenic action as gentle and as constant as possible Thus like *Hay et al* the present author omitted fasting the guinea pigs for the usual 24 or 48 hours before the experiment and thus avoided the acute focal surface necrosis caused by the extreme acid action Fasting is a very potent additive ulcerogenic factor This is apparent from the finding that in experiments using fasting it is possible to induce within a few hours, ulcerations by a histamine dose of 1 2 mg/kg whereas without fasting a considerably higher dose through several days is required

So that the very acute vascular histamine reaction could be avoided the guinea pigs received a small dose of promethazine (2 mg/kg) prior to each injection of histamine This affords a slow induction phase diminishing the vascular part of the peak effect which otherwise follows soon after the histamine injection and which is due int al to the increased volume in the muscles at the site of injection initially entailing an increased pressure and thereby an increased release of histamine The ease with which this phenomenon is obtained may be illustrated by the experiments of Watt who observed an *immediate* vascular reaction in the exposed stomach merely on massaging the site of injection during the experiment

In the same way the present method reduced the stress situation which arises because of the histamine shock which otherwise may follow immediately upon an injection of histamine

It might be objected to the use of promethazine that this substance is *per se* ulcerogenic (4) However this effect requires high doses (more than 50 mg/kg) far exceeding the 2 mg/kg used in the present study Since moreover it has been demonstrated that the ulcerogenic effect of promethazine is due to high doses causing a release of endogenous histamine (5) it is hardly likely that the process of ulceration in the present experiments could have been essentially influenced thereby

As already mentioned one of the controls had two small superficial erosions in the body of the stomach It is not known how often erosions of this magnitude appear spontaneously in guinea pigs as they may be difficult or impossible to detect in an unstained preparation Moreover there have been no re-

ports of studies investigating the spontaneous occurrence of erosions in stained gastric preparations But if the erosions have arisen during the experiment, they might be imagined to have been caused by stress due to the two daily injections, possibly by the weak ulcerogenic effect of promethazine

The classification of the ulceration process into three stages was based upon the following factors (1) Stage I is the most commonly ocurring type of ulceration Owing to the fissure-shaped structure which makes for a quick cover of new epithelium from the edge, it presumably never progresses into the depth, so that a true ulcer can develop direct from this stage Only a small proportion of these ulcerations lead to a stage II ulceration (2) Stage II presents a larger, wider, flat bottomed ulceration It is still in the form of an erosion, as the lamina muscularis mucosae is intact When the central area gastrica undergoes necrosis and is desquamated, a larger, open surface is exposed in which the lamina muscularis mucosae is covered only with a thin layer of connective tissue and which, owing to the greater distance to the marginal zone, is more difficult to cover with a new epithelial layer In this stage, therefore, it is rather likely that the lamina muscularis mucosae will undergo necrosis and the lesion will progress into the depth Even here, only a small proportion of the ulcerations go on to the next stage (3) In Stage III an ulcer arises which as to the surface structure and histologically resembles a human peptic ulcer

As stated, the majority of the ulcerations were situated in the acid-producing corpus-fundus glandular region of the stomach, especially around the greater curvature Thus, their site differed from that seen in human peptic ulcer Statements concerning the location of the ulcerations induced by histamine in experiments reported in the literature have often been very inaccurate, but it appears to be rather flexible, meaning that even minor changes in the experimental conditions may alter the site of the ulcerations Factors which may be operative are the dose of histamine, the duration of the experiment and the frequency of injections, the mode of administration, and possibly other, co-existent supplementary ulcerogenic factors By way of example, it may be mentioned that according to Eagleton & Watt (7) 100 per cent duodenal ulcers arise when a small dose of histamine is injected i p into fasting guinea pigs, whereas 100 per cent gastric ulcers arise when the same dose is administered i m in smaller portions

At present it is not possible to explain why the ulcerations in this study appeared in sulci between the areae gastricae The mucosa in these sites might be imagined to be less resistant due to less mucus production or poorer vascularization, or possibly the ulcerogenic action might have been stronger in these sites due to greater HCl-pepsin production or poorer drainage A local congestive effect might be imagined to occur in the sulci because of poorer drainage, causing less admixture of the produced acid with the food in the stomach This is perhaps supported by the fact that the most ulcerated areas are always observed in that part of the stomach which is most dependent when the animal assumes its normal positions

It is remarkable that such a large proportion of the Stage I and II ulcerations healed during the experiment in spite of the constant histamine load which continued to lead to the formation of new ulcerations This presumably is evidence of the considerable power of regeneration and resistance of the stomach Obviously the rapid regeneration depends highly upon whether the lamina muscularis mucosae, after desquamation of the necrotized areae gastricae in early Stage II, is preserved intact until the bed of the ulceration has acquired a new epithelial layer If the lamina muscularis mucosae degenerates, there will soon occur oedematous swelling of the submucosa, inflammation and thrombosis of the vessels, and the process will inevitably proceed to a Stage III ulcer

Apart from the fact that many ulcerations healed during the experiment, it was also observed that the tendency to ulceration had decreased in the guinea pigs killed last These

animals exhibited many healed ulcerations, but relatively fewer new ones The reason is not clear, but a contributory factor might be that the least resistant areas of the stomach had become ulcerated and had again healed, so that they were left as pylorus-like scar tissue According to several workers (8, 25), such tissue is more resistant to a histamine load than is the normal surface of the body and fundus Indeed, new-formed ulcerations were never seen in this scar tissue in the present study In this way, the weakest areas of the stomach have been rendered more resistant, so that the tendency to a new development of ulcerations ought to be reduced In addition, an adaptation to the histamine effect (6, 1) may have taken place, but normally this requires 8-12 weeks Dmochowski has described a considerably reduced frequency of ulcerations in guinea pigs after 8-12 weeks intraperitoneal adaption to histamine Lastly it might be imagined that the high histamine concentration set up in the thigh muscles at the site of injection could be angiotoxic, causing damage to the vessels and thereby deterioration of the conditions for absorbing the injected histamine

The common use of histamine in experimental research into peptic ulcer must be ascribed to several factors. One of the advantages is that histamine is present physiologically in the human stomach and has a potential possibility of acquiring pathogenetic importance in the development of human peptic ulcer and perhaps even more probably the development of the various forms of acute ulcers Several workers have pointed out the likelihood of histamine being a mediator in stress- and steroid-induced ulcerations in experimental animals For instance *Levine & Senay* (17) have demonstrated that histamine induced increase in the acid production is an important link in the development of stress ulcers in the rat, and *Heisler & Kovacs* (11) have pointed out the pathogenetic contribution of histamine in steroid-induced ulcers. *Schwartz* (21) has published a survey on the role of histamine in various forms of experimental ulcers.

REFERENCES

1 *Ambrus, J L, Ambrus, C M & Harrison, R E* Gastric acid secretion and gastric ulcer formation of rats and guinea pigs Amer J Physiol *173* 95–99, *1953*

2 *Anderson, W & Somann, P D* Histamine gastric ulceration in the guinea pig Some observations on a new method J Pharm Pharmacol *17* 92 97, 1965

3 *Craver, B N* The pharmacodynamics of histamine Ann N Y Acad Sci *50* 1029–1038, 1949

4 *Djahanguiri, B, Sadeghi, D & Hemmati S* The production of acute gastric ulcer by promethazine hydrochloride in the guinea pig Toxicol Applied Pharmacol *12* 568–569, 1968

5 *Djahanguiri, B & Hemmati, M* Histamine endogene et ulceres gastriques prométhaziniques chez le cobaye Therapie *25* 611–614, 1970

6 *Dmochowski, J R & Filip, Z* The influence of intraperitoneal adaption to histamine on the histamine ulcerations in the guinea pig Experientia *20* 220–221, *1964*

7 *Eagleton, G B & Watt, J* The selective production of gastric and duodenal ulceration using histamine Peptic ulcer ed. J Pfeiffer Munksgaard, Copenhagen 1972, pp 34–44

8 *Hale, E H & Grossmann M I* The resistance of recently healed excisional ulcer of the stomach to histamine induced ulcer J Lab Clin Med *34* 228 233 *1949*

9 *Halpern, B N & Martin J* Production experimentale d ulcus et de perforation gastriques par des doses massives d histamine chez le cobaye protegé par des anti histaminiques. C R Soc Biol *140* 830–832 1946

10 *Hay, L J, Varco, R L Code C F & Wangensteen, O H* The experimental production of gastric and duodenal ulcers in laboratory animals by the intramuscular injection of histamine in beeswax Surg Gynec Obstet *75* 170–182, 1942

11 *Heisler S & Kovacs, E M* The possible role of histamine in the cortisone induced gastric acid hypersecretion of the guinea pig Brit J Pharmacol *35* 498–509 1969

12 *Kowalewski, K & Bain C O* Prevention of post histamine gastric ulcers in the guinea pigs by posterior pituitary extract Acta Gastroenterol Belg *17* 539 551 1954

13 *Kowalewski K & Wanmuski C* Effect of exogenous and endogenous histamine on oxygen content and pH of arterial, venous and portal blood in dogs. Arch int Pharmacodyn. *141* 210 215 1963

14 *Kowalewski, K* Vascular factor in the etiology of posthistamine gastric ulcer in guinea

pig Protective action of synthetic vasopressin analogue octapressin Arch int Pharmacodyn *170* 66–73, 1967

15 *Kronberger, L* & *Hafner, H* Über den einfluss des weiblichen sexualhormons auf das ulcus des oberen verdauungstraktes Endokrinologie *49* 185–198, 1966

16 *Landboe Christensen, E* & *Parapat, S B* Low magnification Semimacroscopic-studies on the architectural pattern of the fundic and pyloric mucosal surface in the stomach by the periodic acid Schiff (PAS) staining and clearing technique adapted for mucosal whole mounts. 4 World Congress of Gastroenterology, Copenhagen, abstract 367, 1970

16a *Landboe Christensen, E* & *Parapat, S B* Contrib in , Atlas der Endoskopie und Biopsie des Darmes' Beck, Dischler, Helms & Oehlert (eds) F K Schattauer, Stuttgart New York, 1973

17 *Levine, R J* & *Senay, E C* Histamine in the pathogenesis of stress ulcus in the rat. Amer J Physiol *214* 892–896, 1968

18 *Menguy, R* Effects of histamine on gastric blood flow Amer J Dig Dis. *7* 383–393, 1962

19 *Olovson, T* Experimental induction of acute gastric ulcers with histamine in beeswax J Int Coll Surg *13* 687–696, 1950

20 *Paton, W D M* & *Vane, J R* An analysis of the responses of the isolated stomach to electrical stimulation and to drugs J Physiol *165* 10–46, 1963

21 *Schwartz, J C* Gastric histamine in the pathogenesis of experimental ulcers. Peptic Ulcer, ed J Pfeiffer, Munksgaard, Copenhagen, 1972, p p 190–198

22 *Watt, J* The mechanism of histamine ulceration in the guinea pig Gastroenterology *37* 741–759, 1959

23 *Williams, A W* Acute histamine erosions in the stomach and duodenum of guinea pigs J Path Bact. *63* 465–469, 1951

24 *Williams, A W* Observations on the healing of experimental gastric ulcers in small laboratory animals Brit J Surg *41* 319–326, 1954

25 *Williams, A W* The healing of experimental gastric ulcers. Brit. J Surg *48* 564–572, 1961

Acta path microbiol scand Section A 81, 498–500, 1973

DELAYED HYPERSENSITIVITY DIRECTED AGAINST ARTERIAL ANTIGENS IN THE HYPERTENSIVE DISEASE IN MAN

FINN OLSEN and BIRTE LOFT

Bispebjerg Hospital Medical Department B
(Heads Mogens Bjørneboe, M D , Flemming Quaade, M D , and
Aage Christian Tomsen, M D)

It is demonstrated by means of the leucocyte migration technique that a hypersensitivity of the delayed type seems to be involved in the hypertensive disease in patients suffering from primary essential hypertension In culture chambers containing 50 micrograms/ml of femoral arterial protein, the six of the seven examined hypertensive patients showed a migration index equal to or below 0 8, the lowest being 0 64 The normotensive control persons showed migration indices varying from 0 9 to 1 06 These indices were clearly distinguished from the indices of the hypertensive patients The antigenic substance(s) in the arteries are still unknown

It was demonstrated in a previous paper (*Olsen & Loft*, 1973) that a hypersensitivity of the delayed type was involved in the hypertensive disease in man The method used was the leucocyte migration technique (*Søborg & Bendixen* 1967 *Bendixen & Søborg* 1969) A homogenized aseptic aorta without cadaverosis from a rabbit served as the antigenic substance and the conclusion could be drawn that the delayed hypersensitivity was directed against unknown antigenic substances localized to the aortic wall of the rabbit

The aim of the present work has been to examine whether a delayed hypersensitivity in the hypertensive disease in man also could be demonstrated if homogenized arteries from human subjects served as antigenic substances in the leucocyte migration technique

MATERIAL AND METHODS

A femoral artery without macroscopical atherosclerosis from a man who had been dead for about twelve hours was used for the production of arterial

... was pipetted off from the sediment and the concentration of the protein in the supernatant was measured

As mentioned above the leucocyte migration technique was used The *migration* of the leucocytes from hypertensive patients and normotensive control persons was studied in culture chambers containing an arterial protein concentration of about 16 micrograms and 50 micrograms *per* ml The results were given as the migration index

Received 8 i 73 Accepted 15 ii 73

Requests for reprints should be addressed to *Finn Olsen*, Bispebjerg Hospital, Medical Department B Bispebjerg Bakke 23 DK 2400 Copenhagen NV, Denmark

which is defined as the ratio between the migration area of antigen-containing and antigen free cultures

With a view to evaluating the specificity of the leucocyte migration technique in culture chambers containing femoral arterial proteins the migration was measured in culture chambers containing equal concentrations of protein from homogenized and extracted peritoneal and liver tissue derived from human subjects

Seven patients suffering from *primary* systemic hypertension were examined for a delayed hyper sensitivity directed against antigenic substances in the human femoral artery The blood pressure of the patients varied from 240/140 mm Hg to about 190/120 mm Hg The age of the patients, four men and three women varied from 33 years to 78 years All the patients but one were treated with antihypertensive drugs Hydroflumethiazidum and/or Methyldopum at the time of the study of the leucocyte migration

The six control persons were healthy and normo tensive persons of ages varying from 25 years to 57 years The control group comprised three women and three men

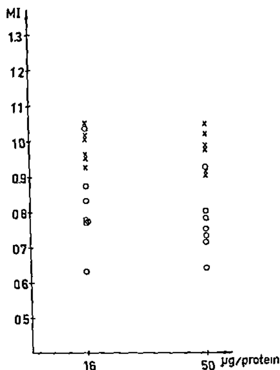


Fig 1 Abscissa Micrograms of femoral protein per ml in the culture chambers. Ordinate Migration index

The figure shows that the migration index of the hypertensive patients (○) is clearly distinguished from the normotensive control persons (×) The majority of the hypertensive patients show a mi gration index equal to or below 0 8

RESULTS

The results of the leucocyte migration given as the migration index, are seen in Fig 1 It appears that the lowest migration indices applying to the hypertensive patients were found if the concentration of the femoral arterial protein in the culture chambers was 50 micrograms At this protein concentration, six of the seven hypertensive patients showed a migration index equal to or below 0 8, the lowest being 0 64 These six patients were clearly distinguished from the normotensive controls If a concentration of only 16 micro grams/ml of femoral arterial protein was used in the culture chambers, only four of the seven hypertensive patients showed migration indices below 0 8, the lowest being 0 63 The latter was observed in the patient who also presented a migration index at 0 64 at a protein concentration 50 micrograms/ml and was suffering from an essential malignant hypertension

The migration index of the normotensive control persons varied from 0 9 to 1 06 at protein concentrations of 16 micrograms/ml and 50 micrograms/ml Accordingly no in hibition of the leucocyte migration was ob served in these cases

If a solution of protein from the peri toneum and the liver was placed in the culture chambers instead of protein from the femoral artery, the migration index from the hypertensive patients varied from 0 9 to 1 04 The method used was in this way specific

DISCUSSION

The described results of the leucocyte mi gration technique demonstrate clearly that at least some patients suffering from essential primary arterial hypertension show a hyper-sensitivity of the delayed type directed against unknown antigenic substances in the arteries

of *man* These results are identical with previous findings (*Olsen* & *Loft,* 1973) in studies in which homogenized aortic tissue of *rabbits* was used as antigen(s) in the leucocyte migration technique Furthermore, it can be concluded that man and rabbits contain common antigen(s) in the arterial walls According to these studies the antigen(s) are probably organ specific, but not species specific which is a well known immunological phenomenon observed in other studies (*Humphrey* & *White* 1970) The results have supported the view that immunological factors of delayed type (*Olsen* 1971, *Olsen* & *Loft,* 1973) seem to be involved in the hypertensive disease in at least some cases of essential arterial hypertension Other studies have shown that a hypersensitivity of the humoral type also seems to be involved in the hypertensive disease (*Ebringer* & *Doyle* 1970 *Olsen* 1972, *Olsen et al*, in press)

REFERENCES

1 *Bendixen, G* & *Soborg, M* A leucocyte migration technique for *in vitro* detection of cellular (delayed type) *hypersensitivity in* man Danish Medical Bulletin *16* 1 6, 1969
2 *Ebringer, A* & *Doyle, A F* Raised serum IgG levels *in* hypertension Brit med J *2* 146–149, *1970*
3 *Humphrey, J H* & *White, R G* Immunology for students of medicine 1970 *Third Edition* P 224
4 *Olsen, F* Evidence for an immunological factor in the hypertensive vascular disease. Acta path microbiol scand Sect A *79* 22–26 1971
5 *Olsen, F* Immunological factors and high blood pressure in man Systemic hypertension and raised levels of immunoglobulins in the serum Acta path microbiol scand Sect A *80* 253–259, 1972
6 *Olsen, F* & *Loft, B* Delayed hypersensitivity and high blood pressure in man Acta path microbiol scand Sect A *81* 145 147 1973
7 *Olsen, F*, *Hilden, M* & *Ibsen, H* Raised levels of immunoglobulin in the serum of hypertensive patients. Acta path microbiol scand Sect A (To be published)
8 *Soborg, M* & *Bendixen, G* Human lymphocyte migration as a parameter of hypersensitivity Acta med scand *181* 247 255, 1967

Acta path microbiol scand Section A 81, 501–506, 1973

CARDIAC RUPTURE IN RECENT MYOCARDIAL INFARCTION

ØYVIND HAVIG

Ullevål Hospital, Department of Pathology, University of Oslo, Oslo, Norway
Head Kristen Arnesen, M D

A prospective, randomized autopsy study of possible pathogenetic factors involved in the mechanism of cardiac rupture following recent myocardial infarction is reported The series consists of 508 cases (279 men and 229 women) Fourteen out of 122 cases with recent myocardial infarction had cardiac rupture This corresponds to a 11 5 per cent frequency of rupture in the infarction group and 2 8 per cent in the total autopsy group Rupture was more common in men than in women, and tended to occur early in the infarction period, 64 per cent of the rupture cases died within 3 days after infarction Attempts at resuscitation was not found to have caused rupture in any case No difference was found in the frequency of diabetes mellitus, hypertension angina pectoris, psychiatric disease or anticoagulant treatment in the ruptured and the non ruptured cases The most characteristic finding in the rupture cases was a thrombotic occlusion of a coronary artery and transmural necrosis in a heart without myocardial scars

In spite of considerable research the mechanism of cardiac rupture is still controversial Most reports on this subject are based upon retrospective studies of clinical records and autopsy protocols

Mechanical forces such as hypertension have been considered as the most important pathogenetic factors by some authors (*Kohn* 1959 *Sugiura* & *Okada* 1970, *Potondi* 1970) Others believe that transmural location of the infarction is the necessary condition for myocardial rupture (*Wessler et al* 1952, *Maher et al* 1956) A combination of these factors has also been pointed out as a possible explanation of cardiac rupture (*Wessler et al* 1952)

This report is based upon a prospective autopsy investigation designed to study pathogenetic factors involving the mechanism of cardiac rupture following recent myocardial infarction

Received 10 xii 72 Accepted 6 ii 73
Requests for reprints should be addressed to Department of Pathology, Ullevål Hospital, Oslo 1

MATERIAL AND METHODS

Selection of cases for the study Five hundred and eight cases (279 men and 229 women) were randomly selected from the autopsy series at the Department of Pathology Ullevål Hospital

Clinical information recorded Age sex, occurrence of diabetes mellitus, hypertension, angina pectoris, psychiatric disease, anticoagulant treatment and smoking habits

Autopsy findings recorded Heart weight, recent myocardial infarction, scars after previous myocardial infarction, coronary thrombosis and degree of coronary atherosclerosis The size and location of myocardial ruptures were noted

Diagnostic criteria of myocardial infarction The diagnosis of recent myocardial infarction was based upon macroscopical and microscopical examination. Only cases in which myocardial necrosis was found at microscopical examination were included in the study

TABLE 1 *Sex Distribution*

Sex	Total no of cases	No of infarcts	No of ruptures
M	279	77	10
F	229	45	4
M + F	508	122	14

Standard sections for histological examination were taken from the left ventricle of all hearts and from the central and peripheral part of all infarcts. Transverse sections from the site of rupture were taken in every case. If in doubt about the existence of infarcts, multiple specimens were taken. The specimens were fixed in 4 per cent formaldehyde solution and the histological sections were stained with haematoxylin-eosin and the Martius-Scarlet Blue (MSB) method. The age of the infarcts were dated according to the criteria established by *Mallory et al* (1939) and *Lautsch & Lanks* (1967).

Statistical method Differences between proportion were tested by any χ^2 test with correction for continuity (*Armitage* 1971).

RESULTS

Fourteen out of 122 patients with myocardial infarction had cardiac rupture (Table 1). This corresponds to a 11.5 per cent frequency of rupture in the infarction group and 2.8 per cent in the total autopsy group (508 cases). There were more men than women with myocardial infarction. More men had myocardial rupture but the sex difference was not statistically significant (Table 1).

The mean age in the group of patients with rupture was higher than in the group of patients with infarction but without rupture especially in males (Table 2).

In most cases the diagnosis of cardiac rupture was made before death and based upon electromechanical dissociation. Attempts at resuscitation were performed in 12 of the 14 rupture cases, and in most of the control cases.

As to the frequency of diabetes mellitus, hypertension, angina pectoris, psychiatric disease, anticoagulant treatment and smoking habits there was no statistically significant difference between the groups of infarcted cases with and without rupture of the heart.

The mean heart weight was 448 g (range 350-630) in the cases of rupture, whereas it was slightly higher in the control cases (489 g range 280-800).

Myocardial scars after previous infarction were found to be significantly more frequent in the cases *without* rupture, and recent coronary thrombosis was found to be more frequent in the cases *with* rupture, though this difference was not statistically significant (Table 4). Coronary atherosclerosis was estimated to be of the same degree in the group of patients with rupture as in the non rupture group.

Seven patients had myocardial rupture in the anterior left ventricle wall and 5 in the posterior left ventricle wall. In one case the rupture was located to the anterior *right* ventricle wall and one patient had rupture of the interventricular septum.

All infarctions were judged to be less than 14 days old and, in 9 of the rupture cases (64 per cent) less than 3 days old (Figure 1). In all rupture cases the myocardial necrosis was *transmural* at the site of rupture. Transverse sections through the infarcted area were taken

TABLE 2 *Age Mean (range)*

Sex	Infarction without rupture (108 cases) mean	range	Infarction with rupture (14 cases) mean	range
M	65.4	(32-88)	70.8	(55-86)
F	71.6	(36-96)	72.5	(64-86)
M + F	67.8	(32-96)	71.3	(55-86)

TABLE 3 *Information Taken from the Clinical Records Number and per cent of Cases*

Clinical informations	The whole group (122 cases)		Infarction without rupture (108 cases)		Infarction with rupture (14 cases)	
	No	Per cent	No	Per cent	No	Per cent
Diabetes mellitus	17	13 9	14	13 0	3	21 4
Hypertension	56	46 0	48	44 4	8	57 1
Angina pectoris	45	36 9	37	34 3	8	57 1
Psychiatric disease	8	6 6	7	6 5	1	7 1
Anticoagulant treatment	24	19 7	21	19 3	3	21 4
Smoking habits (> 10 cig)	26	21 3	20	18 5	6	47 9*

* χ^2 3 04 0 10 > p > 0 05

TABLE 4 *Number and Percentage of Cases with Recent Myocardial Infarction, with and without Myocardial Rupture by Presence of Myocardial Scars and Coronary Thrombosis*

	Infarction without rupture (108 cases)		Infarction with rupture (14 cases)	
	No	Per cent	No	Per cent
Myocardial scars	72	67	4	28*
Coronary thrombosis	44	41	10	71**

* $\chi^2 = 9\ 36$, $p < 0\ 005$
** $\chi^2 = 3\ 56$ $p > 0\ 05$

in only a few of the control cases Thus, the frequency of transmural myocardial necrosis in these cases cannot be stated

Thirteen patients with rupture died suddenly, i e within 15 min of the onset of an acute aggravation whereas one patient gradually became worse during 12 hours prior to death The latter showed a 10 to 12 days old infarction with a small amount of blood within the pericardial sac The transmural tear in the myocardium was very small in this case and covered by a mural thrombus The size of the tear in the other cases ranged from a pinhole to 3 cm long The pathway of the rent usually was circuitous with considerable haemorrhage in the myocardium (Figure 2) The amount of blood in the pericardial sac ranged from 250 to 680 ml

DISCUSSION

The frequency of myocardial rupture in this study 11 5 per cent is within the range 4 7 20 per cent given in the literature (*Edmondson & Hoxie* 1942, *Friedman & White* 1944, *Zeman & Rodstein* 1960, *Stevers* 1966) The different frequencies observed in previous studies may be due to differences in the selection of cases For example, the percentage of patients without myocardial necrosis who die suddenly from arythmia, may not be the same in the various series Such cases are often included in the group of cases called coronary heart disease

In this study the clinical information did not contribute much to elucidate the pathogenesis of myocardial rupture Rupture is said to be associated with age *per se* (*Krumbhaar & Crowell* 1925, *Lunseth & Ruwaldt* 1956) However, the number of cases of rupture probably increases by age because the number of cases of myocardial infarction also increases by age In this and other series (*Wessler et al* 1952) the percentage of patients with rupture is about the same in all ten year age groups

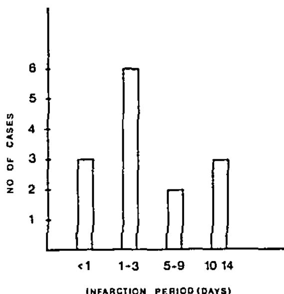


Fig 1 The age of myocardial infarctions at the time of rupture

Rupture was more common in men than in women, though the difference was not statistically significant The figures in the literature however are controversial (*London & London* 1965 *Normand et al* 1965 *Friedman et al* 1971)

Diabetes mellitus is associated with increased coronary atherosclerosis (*Agar* 1962 *Kaffarnik et al* 1971) and with increased frequency of myocardial infarction and myocardial rupture This study showed a slightly higher frequency of diabetes mellitus among the ruptured cases than among the non ruptured The difference is not statistically significant

Myocardial rupture has been observed to be associated with hypertension (*Gans* 1951 *Aarseth & Lange* 1958) even though some authors have not observed such an association (*Kavelman* 1960 *London & London* 1965) In this study, evidence of hypertension was found more often among the ruptured than among the non ruptured cases The difference was not statistically significant On the basis of a large series of 87 cardiac ruptures among 870 cases with coronary occlusion, *Potondi* (1970) stressed the importance of hypertension in combination with small infarcted areas in the pathogenesis of cardiac rupture He claimed that, if only a small portion of the myocardium is infarcted, the muscle of the left ventricle remains strong and a blow-out rupture in the necrotic area may occur This idea is not supported by the present investigation since hypertension was about as common in the control as in the rupture group

This study gives no evidence for any association between pre-existing angina pectoris or mental disease and cardiac rupture Such associations have been stated by some investigators (*Jetter & White* 1944)

Anticoagulant therapy is said to increase the risk of myocardial rupture in cases of myocardial infarction (*Waldron et al* 1954 *Lange & Aarseth* 1958) In accordance with other studies (*Hilden et al* 1961, *London & London* 1965), the present investigation shows no difference in the frequency of anti



Fig 2 Autopsy specimen from a 64 year old man with a three days old anterior infarction resulting in rupture

coagulant treatment in the ruptured and the non ruptured groups

The number of heavy cigarette smokers was found to be higher among the rupture cases than among the controls This has not been observed in other studies The association between smoking and cardiac rupture is obscure

The general view that cardiac rupture most often occurs about one week after infarction i.e when myocardial necrosis is at its maximum (*Wessler et al* 1952), is not supported by the present study In this series, 64 per cent of the patients with rupture died within three days This is in agreement with *Marton & Hagen* (1967) who found that rupture tends to occur early in the infarction period the frequency being highest on the first day Hence, rupture may occur at any stage of the recent infarction Mechanical factors such as paralysis of the infarcted area with dissociated contractions followed by undue stress on certain parts of the infarcted area are probably involved, particularly in cases of clinical hypertension In this respect, rupture of necrotic muscle fibres by constant bending and stretching is a possible pathogenetic factor

Attempts at resuscitation, including external cardiac massage, were performed in most of the cases This procedure may have been a possible cause of rupture However, this could not be verified in any of the cases The rupture most probably occurred before the resuscitation started The great amount of blood found in the pericardial sac must reasonably have been caused by a beating heart Secondly, a more extensive bleeding in the myocardium would have been expected if the resuscitation had traumatized the heart to such a degree as to cause rupture Thirdly the frequency of rupture in this series does not exceed the frequency found in most other series in which resuscitation was not attempted

Rupture of the right ventricle is rare, about 10 per cent of the rupture cases (*Krumbhaar & Crowell* 1925) In the present study, one patient with right ventricular hypertrophy showed rupture of the right ventricle Pulmonary hypertension may have been a pathogenetic factor in this case as stated by others (*Wade* 1959)

The relatively low frequency of scar tissue after previous myocardial infarction in cases of cardiac rupture following recent myocardial infarction (one out of four cases), have been reported by many authors (*Edmondson & Hoxie* 1942, *London & London* 1965) This corresponds well with 4 out of 14 cases in the present study On the other hand, 67 per cent of the controls showed scar tissue after previous infarction How scar tissue may prevent rupture in a new episode of infarction is obscure

This study showed a higher frequency of recent coronary thrombosis in the rupture cases The results confirm observations made by other investigators (*London & London* 1965)

It may be concluded from the present study that there is no difference in the frequency or diabetes mellitus, hypertension, angina pectoris, psychiatric disease or anticoagulant treatment in the ruptured and the non ruptured groups. The most characteristic finding in the rupture cases was a thrombotic occlusion of a coronary artery and transmural myocardial necrosis in a heart without myocardial scars

The author expresses his sincere gratitude for the financial support of this study obtained from *Nasjonalforeningen, Det norske råd for hjerte og karsykdommer*

REFERENCES

1 *Aarseth, S & Lange, H F* The influence of anticoagulant therapy on the occurrence of cardiac rupture and hemopericardium following heart infarction I A study of 89 cases of hemopericardium (81 of them cardiac ruptures) Amer Heart J *56* 250–256, 1958

2 *Agar, J M* Silent myocardial infarction in diabetes mellitus Med J Aust *49* 284–285, 1962

3 *Armitage, T* Statistical methods in medical research Blackwell scientific publications Oxford and Edinburgh 1971, p 135

4 *Edmondson, H A & Hoxie H J* Hypertension and cardiac rupture Clinical and patho-

logical study of 72 cases in 13 of which rupture of interventricular septum occurred Amer Heart J 24 719–733, 1942

5 *Friedman, S & White, P D* Rupture of heart in myocardial infarction Experience in a large general hospital Ann Int Med *21* 778–782, 1944

6 *Friedman, H S, Kuhn, L A & Katz, A M* Clinical and electrocardiographic feature of cardiac rupture following acute myocardial infarction Amer J Med *50* 709–720, 1971

7 *Gans, R H* Acute myocardial infarction with rupture of ventricle Amer Heart J *41* 332–339, 1951

8 *Hilden, T, Iversen, K, Raaschou, F & Schwartz, M* Anticoagulants in acute myocardial infarction Lancet *2* 327–331, 1961

9 *Jetter, W W & White, P D* Rupture of heart in patients in mental institutions. Ann Int Med *21* 783 802, 1944

10 *Kaffarnik, H, Lingelbach, H, Gassel, W D & Heimsoth, V* Häufigkeit diabetischer Stoffwechselstörungen bei Herzinfarktpatienten Parallele Untersuchungen mit zwei Testmethoden Dtsch med Wschr *96* 1659 1661 1971

11 *Katzelman D I* Myocardial rupture a study in non psychotic and psychotic patients. Canad Med Ass J *82* 1105–1107 1960

12 *Kohn R M* Mechanical factors in cardiac rupture Amer J Cardiol *4* 279 281 1959

13 *Krumbhaar E B & Crowell, C* Spontaneous rupture of the heart Amer J Med Sci *170* 828–856 1925

14 *Lange, H F & Aarseth, S* The influence of anticoagulant therapy on the occurrence of cardiac rupture and hemopericardium following heart infarction II A controlled study of a selected treated group based on 1044 autopsies Amer Heart J *56* 257 263 1958

15 *Lautsch, Elizabeth V & Lanks K W* Pathogenesis of cardiac rupture Arch Path 84 264–271 1967

16 *London, R E & London, S B* Rupture of the heart, a critical analysis of 47 consecutive autopsy cases Circulation *31* 202 208 1965

17 *Lunseth, J H & Ruwald, M* Pathogenesis of cardiac rupture due to myocardial infarction Dis. Chest *30* 499–507, 1956

18 *Maher, J F, Mallory, G K & Laurenz, G A* Rupture of the heart after myocardial infarction New Engl J Med *255* 1–10 1956

19 *Mallory, G K, White, P D & Salcedo Salgar, J* The speed of healing of myocardial infarction A study of the pathologic anatomy in 72 cases. Amer Heart J *18* 647–671 1939

20 *Marton, P F & Hagen, T* Cardiac rupture in myocardial infarction J of the Oslo City Hospitals *17* 37–54, 1967

21 *Normand, P J, Perrin, A, Plauchu G & Froment, R* Les ruptures parietales et septales *au cours des infarctus myocardiques* (Apropos de 30 observations anatomo-cliniques) Arch. Mal Coeur *58* 1311–1336, 1965

22 *Potondi, A* Pathomechanism of spontaneous cardiac rupture following infarction Z. Rechtsmedizin *68* 7–10, 1971

23 *Sievers, J* Cardiac rupture in acute myocardial infarction Geriatrics *21* 125–130, 1966

24 *Sugiura, M & Okada, R* Myocardial rupture in old age Geriatrics *25* 130–139 1970

25 *Wade, W G* The pathogenesis of infarction of the right ventricle Brit Heart J *21* 545–554, 1959

26 *Waldron B R, Fennell R H Jr, Castleman B & Bland, E F* Myocardial rupture and hemopericardium associated with anticoagulant therapy New Engl J Med *251* 892–894 1954

27 *Wessler, S, Zoll, P M & Schlesinger, M J* The pathogenesis of spontaneous cardiac rup-

28

Amer Arch int Med *105* 431–443 1960

Acta path microbiol scand Section A 81, 507 522 1973

ELECTRON MICROSCOPIC OBSERVATIONS ON FRACTURE REPAIR IN THE RAT

GUSTAV GOTHLIN

Departments of Pathology at Sabbatsberg Hospital and Orthopedic Surgery at Norrbackainstitutet, Karolinska Institutet Medical School, Stockholm, Sweden

The morphology of the different cell types in 'primary' callus obtained by fracturing the femur shaft of rats is described in this investigation which is based on electron microscopic observations of undemineralized callus tissue obtained at various intervals after fracturing The different cell types could be divided into two main groups, one consisting of cells with predominant matrix manufacturing properties, and one with phagocytic (or matrix resorbing) function Macrophages were not only present during early callus formation but were also observed in the callus until the late mineralized stages and may be of importance for remodeling of the tissue at the late intervals Large electron dense mitochondrial granules were found in chondrocytes, osteoblasts, osteocytes, and osteoclasts located close to calcified matrix

Electron microscopic studies of the ultrastructure of fracture callus are few and fragmentary This type of tissue has been used in ultrastructural work to describe and identify certain types of bone cells, such as osteoclasts (13) and osteoblasts-osteocytes (6, 7, 8, 9) *Aho* (1) was the first to investigate callus tissue ultrastructurally in different stages of fracture healing The observations in *Aho's* study (1) were made on rats, and callus tissue was obtained and prepared for ultrastructural study at 7 intervals between 1 and 30 days after fracturing Although the aforementioned investigation represents a valuable pioneer work, certain weaknesses in the preparatory procedures, especially the use of methacrylate as embedding medium, made the proper interpretation and evaluation of the pictures difficult, and some of the conclusions drawn may have to be reconsidered in the light of results obtained with more recently developed techniques

The present study forms part of a series of interrelated investigations on rat fracture callus using morphological, histochemical and autoradiographic methods applied on the ultrastructural level In this investigation certain basic ultrastructural data are presented regarding the cells occurring during different stages of fracture healing The main aim of the investigation was thus to create a base line for further studies by supplying data regarding the fine structure of properly preserved cells in the callus and their interrelations with one another Furthermore, the appearance of the cells in calluses of variable age was elucidated

Received 2 ii 73 Accepted 10 ii 73

Request for reprints should be addressed to Dr *Gustav Gothlin*, Patologiska institutionen, Sabbatsbergs sjukhus Box 6401, S-11382 Stockholm Sweden

MATERIALS AND METHODS

. . . . .

dy A complete fracture of the shaft of the femur was produced *digitally* or *by means of a rubber* dressed haemostat under light ether anesthesia As a rule, both femurs were fractured Rats were sacrificed by decapitation in groups of 2 or 3 on days 2 7, 9, 10, 12 15, 17, 19, 20, 23, 25, 27, 31, 36 and 40 after the fracture The fractures were immediately exposed and dissected free for gross examination The stability was tested digitally, and samples *from the fracture sites and calluses for electron* microscopic study were excised with a razor blade both *from the fracture gap* and the periosteal areas. The excised tissues were carefully trimmed to cubes with a side of ~ 1 mm under a drop of fixative and were subsequently immersed in the same fixative Fixation was performed at 0-4° C in 2 per cent *s* collidine buffered osmium tetroxide *(OsO_4) (pH 7 4) for 1 5 2 hours, or in 1 5 per* cent purified glutaraldehyde buffered with 0 1 M cacodylate buffer for 24 to 48 hours. Glutaraldehyde-fixed tissues were washed in cacodylate buffer and were then postfixed in collidine buffered OsO_4 overnight All the tissues were dehydrated in alcohol solutions of increasing strength and propylene oxide, and were embedded in Epon epoxy resin Approximately 1 μ thick Epon *sections prepared on* glass knives were stained with alkaline toluidine blue for orientation in the blocks. Suitable areas were *trimmed out for thin sectioning* The thin sections were stained with lead citrate and/or uranyl acetate and were studied in a Siemens Elmiskop I electron microscope

RESULTS

The cells *taking part* in callus *formation* at different intervals after the fracture show a highly variable fine structure Classification of these cells was made possible by comparison with results from other calcifying tissues and by identification of apparent transitional forms between the different types

In this way, two main groups of *cell* types could be distinguished *(A) Matrix producing cells, constituting precursor cells* ["osteoprogenitor cells", "reticular' or mesenchymal cells, *Scott's* type A cells (26)], fibroblasts chondroblasts, chondrocytes, osteoblasts, osteocytes, and endothelial cells (17), in addition to transitional forms (Figs 1-9), and *(B) cells unassociated with matrix production*, consis*ting of precursor cells [monocytoid cells, Scott's* type B cells (26)], leucocytes, histiocytes, macrophages (chondroclasts, preosteoclasts), and osteoclasts (Figs 10-18)

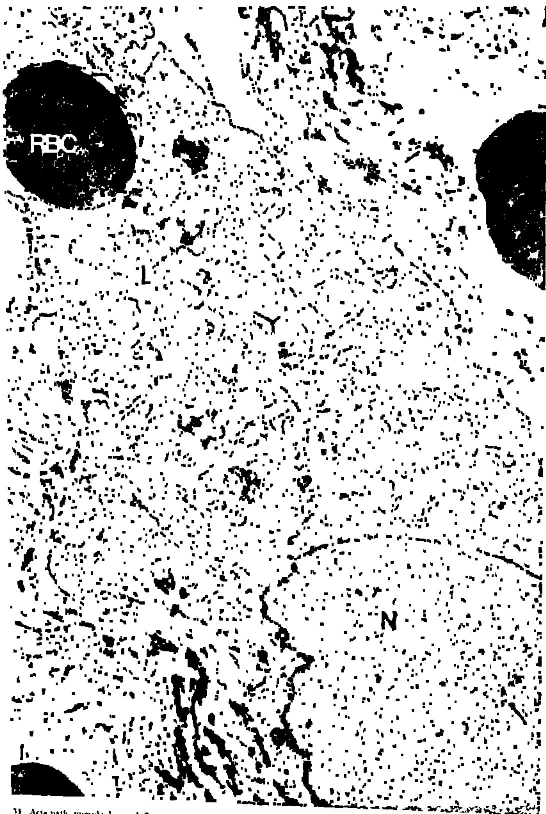
A characteristic feature of the cells in *group A* is the occurrence of a well-developed endoplasmic reticulum (Fig 5) This tendency becomes more conspicuous in the mature forms of the cells, where the endoplasmic reticulum membranes are stacked together tightly to form systems of lamellar or cisternal configu*ration The Golgi apparatus is generally more* sparse than in the group B cells, although in mature forms it can have a rather complicated, sometimes cup shaped appearance (Fig 9) The mitochondria are not as abundant as in the group B cells of corresponding differentiation Lysosomes are usually more sparse and show different morphological appearances in group A as compared to group B cells

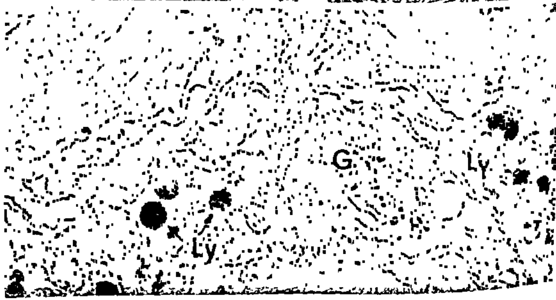
Group B essentially consists of cells with *phagocytic or matrix-resorbing properties* and their apparent precursor forms (*cf* Fig 16) Among these cells there were many immature forms which were difficult to specify with regard to cell of origin However, common features were the sparse formation of endoplasmic reticulum membranes, the abundance of Golgi systems, and the occurrence *of numerous* mitochondria Another characteristic *was the abundance of lysosomes*, histochemically proven by their content of acid phosphatase (15) The cell surface often had a villous appearance Some of the macrophage like cells tended to show morphologic differentia

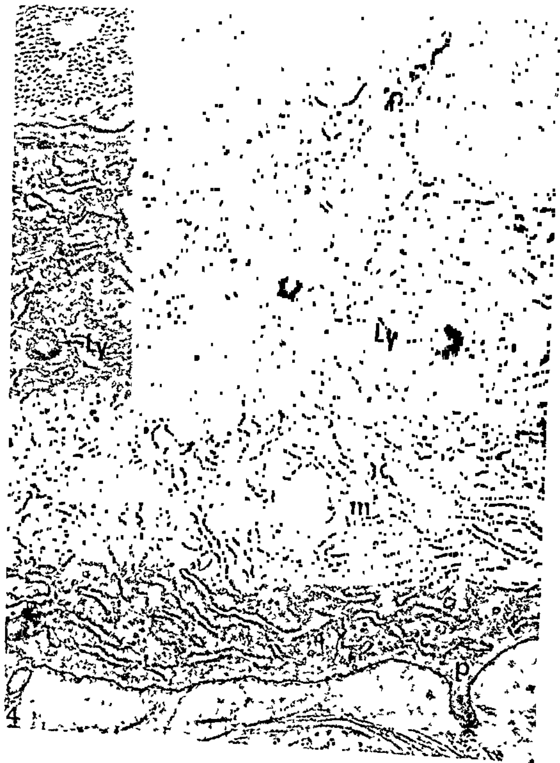
---

Note All the sections depicted in the figures have been stained with uranyl acetate and lead citrate

Fig 1 2 days gap The picture illustrates a portion of a cell with abundant cytoplasm containing moderate numbers of meandering cisternae of rough surfaced endoplasmic reticulum numerous clusters of ribosomes in the ground substance and several lipid droplets (L) of variable size Mitochondria are few N nucleus, RBC, red blood cells This cell is interpreted as representing an immature osteogenic cell (preosteoblast) (Scott s type *A cell*) × 12 500


RBC
L
N


G
Ly
Ly
3



33*



Fig 5 14 days periost A cell with irregularly undulating plasma membrane and abundant rough surfaced endoplasmic reticulum showing a clear
region (G) *is comparatively large and cisterna*
rounded by numerous vesicles L lip d droplets
the vicinity of the cell Although it bears strong resemblance to a f broblast the irregular con ou the plasma membrane and the abundance of endoplasmic reticul m tending to form parallel c sternae suggests that it represents an interm ediary form between fibroblast and osteoblast × 11 500

Fig 2 7 days gap This cell contains h gh concentrations of haphazardly oriented cisternae of rough surfaced endoplasmic reticulum Two Golgi regions (G) are present in the f eld illustrated Small coated ves cles (arrows) are arranged in the per phery of these regions and are also occasionally seen in other areas of the cytoplasm Ly sn all lysoson e near the Golgi apparatus This cell is considered to be a well developed fibroblast × 22 400

Fig 3 31 days gap *Portion of fibroblast l ke cell* resembling the one illustrated in Fig 2 Note the cup shaped *appearance of the Golgi apparatus* (G) On the convex side of the Golgi apparatus *there are several* vesicles some of which appear to be coated Ly lysosomes × 13 300

Fig 4 14 days periost A cell w th fa rly abundant cisternae of rough surfaced endoplasm c ret culum d stributed rather haphazardly in the cytoplasm The pla ma membrane forms rregular protrus ons (p) into areas with collageno s extracellular matrix A mitochondrion (m) is devo d of dense matrix granules The *Golgi* reg ons are small and poorly developed Ly lysosomes Although th s cell shows similar cytoplasmic *features* as f broblasts the occurrence of protrusions and the tendency toward *scattering of small areas* of Golgi reg ons and loss of contact between plasma membrane and collagen (*lower part* of the picture) suggests that this may represent an intermediary form bet een f broblast and chondroblast × 20 700

tion in the direction toward osteoclasts, and apparent transitional forms ("preosteoclasts") between these two cell types were encountered These transitional forms were characterized by abundance of mitochondria, presence of a villous border, and numerous lysosomes and subplasmalemmal vesicles and vacuoles

The fine structure of most of the cell types forming the callus (*fibroblasts, histiocytes, leucocytes, chondroblasts, osteoblasts, osteoclasts, and others*) has been studied in considerable detail in other types of tissues (5 8, 10 12, 22-25, 28), and are illustrated in the figures, hence, they need no further comment. However, a few other cell forms—especially the immature types of cells (Fig 14) constituting the invading complex of early callus—have previously not been adequately defined or have defied accurate idenfication The same is the case of early forms of osteoblasts (Fig 1) and osteoclasts, and transitional forms between fibroblasts and chondroblasts (Fig 4)

Occurrence of the Various Cell Types in Relation to Different Stages of Fracture Healing

Undifferentiated cells, constituting entities which can be referred either to the type A cells (Fig 1) or type B cells (Fig 16) of Scott (26) formed—together with immature fibroblasts, leucocytes, histiocytes and macrophages—a dominating feature of callus tissue during the first 5 6 days after fracturing They were subsequently replaced by more specialized osteogenic cells However, during the whole span of callus remodeling—up to 40 days after fracturing—such indifferentiated cells were occasionally encountered, especially in the fracture gap area, and often in connection with capillary "sprouts"

Fibroblasts (Figs 2 and 3) were present in abundance, especially in the periosteal parts of the callus during the first 12-15 days post fracturing After 10 days, however, they began to decrease in number, and later (up to the 15th–17th day of callus formation) they were continuously being substituted by *chondroblasts* as the most common cell type of periosteal areas The chondroblasts first appeared in immature form at the 7 day interval, and developed to form typical mature chondroblasts (12) 12 days after fracturing However, both fibroblasts and chondroblasts were encountered in small numbers in fibrocartilaginous islands of callus even at late stages (30 40 days after fracture) when the callus was consolidated

Osteoblasts in immature forms (Fig 5) were first seen at the 8 day interval when they could be identified by their histochemical properties (14) In mature forms with an ultrastructural appearance classically attributed to osteoblasts (5, 8), they were found from the 14th day of callus formation (Fig 6) In periosteal areas of the callus they constitute the most conspicuous cell type from days 21-23

With regard to cells *"unassociated with matrix production"* there were abundant leucocytes, immature monocytic cells, and histiocytes in early stages of fracture healing Macro

Fig 6 15 days, periost An osteoblast from an area of commencing calcification The Golgi apparatus is rather well developed The cytoplasm contains mitochondria with numerous electron dense granules closely related to the cristae note coated vesicles (cv) closely associated with the Golgi apparatus. AV, autophagic vacuole × 44,800

Fig 7 21 days, periost A cell located in an area with calcification in the matrix The cytoplasm contains abundant rough surfaced endoplasmic reticulum and mitochondria with pale matrix and numerous relatively large matrix granules (arrows) The inset shows 2 such granules at higher magnification Note that the granules are closely associated with mitochondrial cristae G, Golgi apparatus C, calcified area in the matrix This cell is interpreted as an osteoblast × 15,000 inset × 45 000

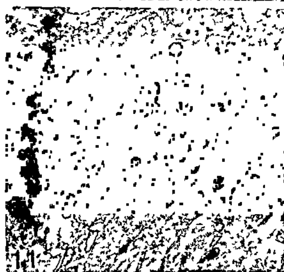
Fig 8 33 days, gap A mitochondrion with several matrix granules and cisternae of dilated rough surfaced endoplasmic reticulum in an osteoblast × 33,000

Fig 9 33 days, gap From an osteoblast completely surrounded by calcified extracellular matrix The Golgi apparatus is cup shaped, and there are several vesicles near the concave side. Ly, lysosomes. × 18 000



AV
CV
CV


G
LY
LY


11


12


13

14
RBC
15
L
Mv
Ly
Ly
L
L
L
Ly
Ly
ER
G



Fig 16 12 days, gap This picture illustrates a cell containing rather numerous mitochondria, some lysosomes (Ly) and moderately large Golgi areas (G) The nature of this cell is not completely clear It may represent the type B cell described by Scott × 18,000

Fig 10 33 days, gap This picture illustrates the appearance of the cytoplasm in an osteoclast Note the large numbers of mitochondria and vesicles Rough surfaced endoplasmic reticulum is rather inconspicuous × 12,000

Fig 11 33 days, gap Superficial area of osteoclast Calcified material represented by densely packed small spikes are present on the surface of the cell (to the left) and in many invaginations in the apical cytoplasm × 13,500

Fig 12 28 days, periost Osteoclast with small vacuoles containing clusters of spiculae similar to those shown in Figure 11 × 23,000

Fig 13 33 days, gap An osteoclast with mitochondria containing numerous large matrix granules The matrix of these mitochondria is poorly preserved, but in some places remnants of cristae can be seen × 14,000

Fig 14 2 days, gap An immature cell surrounded by cell debris and a red blood cell (RBC) × 14 000

Fig 15 2 days, gap A macrophage with irregular plasma membrane forming small microvillous protrusions (Mv) The cytoplasm contains numerous lysosomes (Ly) and many lipid droplets (L) G, Golgi apparatus Rough surfaced endoplasmic reticulum (ER) is inconspicuous × 18,200





Fig 17 14 days, gap A macrophage (MA) containing numerous large lysosomes is located near a capillary × 14,000

Fig 18 20 days, periost A macrophage containing several lysosomes with variably shaped inclusions is illustrated × 12,500

phagelike cells (Figs. 15, 17 and 18) were uni- or multinuclear and of variable size, they were encountered in the fracture gap throughout the whole process of callus remodeling Immature forms of these cells occurred during the first 10 days. *In late stages of callus formation* when calcification had begun (10-15 days and later), large macrophages often containing several nuclei were observed in the close vicinity of calcified portions of matrix They often embraced bone fragments, although apatite crystals were never clearly demonstrated to be enclosed within the numerous and complex folds formed by the surface projections directed against the matrix Osteoclasts (Figs 10, 11, and 12) were seldom found before day 14 but later increased *in number to become most commonly* encountered on days 25-30 after fracturing They were usually found in the fracture gap and were often associated with bone walls or bone fragments in the matrix. Calcified portions of matrix were sometimes totally enclosed in the cytoplasm of huge osteoclasts.

Mitochondrial Matrix Granules

A striking feature of those osteoblasts, osteocytes and osteoclasts which were located in the close vicinity of mineralized matrix surfaces was the presence of numerous large electron dense granules ("matrix granules") (18-21), often associated with the cristae of the mitochondria (Figs 6 8, and 13) At high magnification these granules appeared to be composed of short, disorderly aggregated rods and angulated crystalline structures

DISCUSSION

Despite the increasing number of published observations, there are many unsolved problems and areas of controversy regarding the cellular events and metabolic properties of osteogenic cells, as they are reflected in the ultrastructure of the individual cells during ossification processes Most of the data related to the ultrastructure of osteogenic tissues have been provided by using the epiphyseal growth plate as a model for bone differentiation (2, 3, 27) By comparison with previous studies of other types of bone forming tissues it was possible to identify most of the cells in the callus It appeared that these cells had a fine structure very similar to that observed in the other tissues By comparing the cell population —at brief intervals—during subsequent stages of callus formation identification of transitional forms and immature elements became possible In this way this ultrastructural analysis of fracture callus provided new information regarding morphological modulations associated with the maturation of osteogenic and phagocytic cells

The findings during early stages of fracture repair supported *Scott's* idea (26) that there are two ultrastructurally different groups of cells during early osteogenesis presumed to represent precursors of osteoblasts and osteoclasts, respectively Thus one population of cells was characterized by a high nuclear cytoplasmic ratio, elongated shape perivascular localization, abundance of endoplasmic reticulum, poorly developed Golgi elements and few mitochondria These cells appeared to correspond to *Scott's* type A cells Other apparently immature cells showed presence of a well developed Golgi apparatus, and contained many mitochondria and dense bodies. These cells appeared to correspond to *Scott's* type B cells

According to the classical definition given by *Scott and Pease* (27), osteoblasts are arranged in a single layer along the calcified cartilaginous matrix This is the case in the rather regulary formed tissue of the epiphyseal plate In fracture callus, however, there is marked delay in calcification, which often begins with diffusely spread "nodulus" like foci (4) in the matrix at some distance from the cells This makes identification of the osteoblasts more difficult in the callus than in the epiphyseal plate However, in the present study comparisons with the fine structural localization of alkaline phosphatase (14) in the osteoblasts facilitated the definition of these cells

In the early stages of callus formation (8 to 15 days) a very heterogeneous population of cells was found in the tissue Fibroblasts and osteoblasts and transitional forms between these two cell types were frequently encountered Chondroblasts and chondrocytes also occurred quite commonly and were easily identified Likewise, macrophages and their monocytoid precursors were *abundant* during the early stages of fracture healing Presence of the different types of phagocytic cells observed in this study was not emphasized in previous work on callus tissue The present findings not only have demonstrated that these cell types occur in abundance at the early stage of callus formation *but* also that they persisted in the *later stages* where calcification was conspicuous and solid callus was formed These findings seem to support the notion that macrophages and related cells are important elements during the remodeling of bone tissue, their properties appeared to be closely related to those of *osteoclasts* The ultrastructural features of these two cell forms also suggest that they are functionally closely related

One of the striking features of bone cells

observed in studies of sections of undemmera lized bone is the presence of electron dense granules associated with the cristae of the mitochondria These so-called mitochondrial granules have been described in chondrocytes in the epiphyseal growth plate as well as in osteoblasts, osteocytes and osteoclasts in other bone tissues, cells with mitochondria contai ning such granules have regulary been located in the vicinity of calcified matrix (18 21) Calcium has been presumed to be a consti tuent of these granules by autoradiography with ⁴ Ca and by microincineration (19, 21) These observations have been taken to support the concept that the mitochondrial granules are in some way related to the calcium concentrating or calcium transporting activities of the cells In the present study electron dense mitochondrial granules were observed in cells located near calcified matrix, these cells included chondrocytes, osteoblasts, osteo cytes, and osteoclast Such observations have, to the best of our knowledge, not been reported previously in callus tissue during fracture repair

The finding of mitochondrial granules in chondrocytes, taken together with the fact that these cells have alkaline phosphatase activity (14) supports the thesis that chondrocytes should be added to the list of possible pre cursors of bone matrix manufacturing cells (16)

This investigation has been supported by grants from *Konung Gustav V s åttioårsfond*

The assistance of Miss *Silwa Vengarelli*, Mrs *Britt Marie Åkerman* and Mr *Magnus Norman* is gratefully acknowledged

REFERENCES

1 *Aho A J* Electron microscopic and histolo gical observations on fracture repair in young and old rats Acta path microbiol scand, Suppl *184* 1 95 1966

2 *Anderson C E & Parker, J* Invasion and resorption in enchondral ossification J Bone Jt Surg (Amer) *48* 899 914, 1966

3 *Anderson C E & Parker, J* Electron microscopy of the epiphyseal cartilage plate A critical review of electron microscopic observations on enchondral ossification Clin Orthop *58* 225–241, 1968

4 *Bonucci, E* Fine structure of and histochemistry of ' *Calcifying globules* ' *in epiphyseal car* tilage Z Zellforsch *103* 192 217, 1970

5 *Cameron, D A* The fine structure of osteoblasts in the metaphysis of the tibia of the young rat *J biophys biochem cytol 9 583*–595, 1961

6 *Cameron, D A* The formation of collagen fibrils in bone In Electron microscopy Proceedings of the *Fifth Internat Congr for* Electron Microscopy, Philadelphia, August 29th to September 5th, 1962 (*S S Breese, Jr*, ed), vol 2, p QQ 8 New York and London, Academic Press *1962*

7 *Cameron, D A* The fine structure of bone and calcified cartilage Clin Orthop *26* 199 228, 1963

8 *Cameron, D A* The fine structure and func tion of bone cells In The biological basis of medicine, (*Bittar, E E & Bittar, E*, eds), vol 3, pp 391–423 London and New York Academic Press, *1969*

9 *Cameron, D A*, *Paschall, H A & Robinson, R A* The ultrastructure of bone cells In Bone Biodynamics, (*Frost, H M*, ed) pp *91–104*, Boston, Little, Brown, *1964*

10 *Chapman, J A* Fibroblasts and collagen Brit med bull *18* 233 237, 1962

11 *Dudley, H R & Spiro, D* The fine structure of bone cells J Biophys Biochem Cytol *11* 627 649, 1961

12 *Godman, G C & Porter, K R* Chondrogene sis studied with the electron microscope J Biophys Biochem Cytol *8 719–760, 1960*

13 *Gonzales, R & Karnovsky, M J* Electron microscopy of osteoclasts in healing fractures of rat bone J Biophys Biochem Cytol *9 299 316, 1961*

14 *Gothlin, G & Ericsson J L E* Fine structu ral localization of alkaline phosphomonoester ase in the fracture callus of the rat Israel J Med Sci *7 488–490, 1971*

15 *Gothlin, G, Ericsson, J L E, Arborgh B & Helminen, H J* Unpublished results *1972*

16 *Holtrop, M E* The origin of bone cells in *endochondral ossification 3rd Europ Symp* on Calcif Tiss (*H Fleisch, H J J Blackwood and M Owen* eds) pp 32–36, 1966

17 *Mankin, H J* Osteogenesis in subchondral bone of rabbits, J Bone & Joint Surg (Amer) *46* 1253–1261 1964

18 *Martin J H & Matthews, J L* Mitochondrial granules in chondrocytes Calcif Tissue Res. *3* 184–193, 1969

19 *Martin, J H & Matthews, J L* Mitochondrial granules in chondrocytes, osteoblasts and osteocytes an ultrastructural and microincineration study Clin Orthop *68* 273–278, 1970

20 *Mattheus, J L* Ultrastructure of calcifying tissues (Amer) J Anat *129* 451–457, 1970
21 *Mattheus, J L* & *Martin, J H* Intracellular transport of calcium and its relationship to homeostasis and mineralization An electron microscope study American J Med *50* 589–597, 1971
22 *Robinson, R A* Morphology of osteoblasts, osteocytes, and osteoclasts with remarks about their function In Tumors of Bone and Soft Tissue A collection of papers presented at the eigth annual clinical conference on cancer, 1963 at the University of Texas, M D Anderson Hospital and Tumour Institute Houston, Texas Year Book Medical Publishers Inc, Chicago, 1965
23 *Robinson, R A* & *Cameron, D A* Electron microscopy of cartilage and bone matrix at the distal epiphyseal line of the femur in the newborn infant J Biophys. Biochem Cytol. 2 253–260, 1956
24 *Robinson, R A* & *Cameron, D A* Bone In Electron Microscopic Anatomy, (Kurtz, S M ed) pp 315–340, New York, Academic Press, 1964
25 *Ross, R* The connective tissue fiber forming cell Chap 1 in Treatise on collagen, vol 2 (Biology of collagen) (Gould, B S, ed) part A, p 182, London and New York, Academic Press, 1968
26 *Scott, B L.* Thymidine ^{3}H electron microscope radioautography of osteogenic cells in the fetal rat J Cell Biol *35* 115–126, 1967
27 *Scott, B L* & *Pease, D C* Electron microscopy of the epiphyseal apparatus. Anat. Rec. *126* 465–495, 1956
28 *Silberberg, R* Ultrastructure of articular cartilage in health and disease Clin. Orthop *57* 233–257, 1968

Acta path microbiol scand Section A 81, 523 542, 1973

ELECTRON MICROSCOPIC STUDIES ON THE UPTAKE AND STORAGE OF THORIUM DIOXIDE MOLECULES IN DIFFERENT CELL TYPES OF FRACTURE CALLUS

GUSTAV GOTHLIN and JAN L. E ERICSSON

Departments of Pathology at Sabbatsberg s Hospital and Orthopedic Surgery at Norrbackainstitutet, Karolinska Institutet Medical School, Stockholm, Sweden

The pattern of uptake and storage of exogenous, electron dense macromolecules—thorium dioxide particles—was studied in an attempt to classify and elucidate the functional significance of the different cell types observed during formation and remodeling of fracture callus in the rat. Two major groups of cells were distinguished on the basis of ability for endocytosis and storage (*a*) Those mainly specialized for phagocytosis (monocytes, histiocytes, macrophages and osteoclasts) and, in addition, immature fibroblasts, and (*b*) fibroblasts, chondroblasts, chondrocytes osteoblasts and osteocytes (known to participate in the elaboration of extracellular collagenous material) The latter group of cells may—in addition to their bone forming capability—also have limited degradative functions since they are able to perform heterophagy Occurrence—in monocytes, histiocytes, macrophages, and immature fibroblasts—of collagen like fibers in thorium dioxide containing lysosome like bodies suggests a role of these cells in the remodeling of the callus and the resorption of the matrix In all the different cell types of the callus, marker molecules were stored for long periods of time indicating lacking or low ability for exocytosis of lysosomal content, and stability and longevity of the lysosomes. The findings suggested that uncoated Golgi associated vesicles in the different osteogenic and phagocytic cells of the callus were derived from the plasma membrane (and represented endocytotic vesicles) while coated Golgi vesicles' might represent primary lysosomes

Thorium dioxide molecules are electron dense and large enough to be readily visualized in the electron microscope (7, 8, 9) In moderate concentrations these molecules do not seem to be harmful to cells and tissues in experimental animals—nor to cells in tissue culture (5, 6, 8) These molecules have been found to be useful as tracers in fine structural studies of endocytosis and storage of macromolecules in various cell types (5, 6, 8, 9).

Electron microscopic studies on the uptake of Thorotrast® (thorium dioxide molecules in suspension) in mesothelial, reticuloendothelial, and hepatic parenchymal cells of the rat and other species—as well as in tissue cultured cells—have revealed that the intracellular

Received 15 i 73 Accepted 16.ii.73

Requests for reprints should be addressed to Dr *Jan Ericsson*, Patologiska institutionen, Sabbatsbergs sjukhus, Box 6401, S 11382 Stockholm, Sweden

distribution of marker molecules in such cells seems to follow a general pattern (7, 18, 19) Initially the electron dense particles are localized in cytoplasmic vacuoles ("phagosomes", phagocytic or endocytotic vacuoles) formed by pinched off invaginations of the plasma membrane Later they accumulate in secondary lysosomes ("phagolysosomes"), and seem to be retained in these structures for long periods of time (5, 6, 8, 9)

Recent work has described the way of uptake of thorium dioxide molecules by osteoclasts in fracture callus (14) The mode of uptake and distribution of Thorotrast® in other cell types of fracture callus is also of interest for several reasons For instance, studies of Thorotrast®-exposed cells can be expected to supply information concerning differences in the ability for endocytosis among the various cell types in the callus and in this way provide evidence for functional differences between the cells Such studies should also be of value in the elucidation of the 'intracellular digestive tract" of the cells and its relation to other cytoplasmic structures They should further aid in clarifying questions related to the storage capacity and turnover of lysosomes and related structures in the cells Finally, if characteristic patterns of distribution of label in the different cell types should be revealed, and these patterns should turn out to be retained for long periods of time the studies could also be expected to provide new information concerning the morphogenesis of the cells in the callus

In the present study the uptake and storage of thorium dioxide molecules by the different cell types in fracture callus has been investigated in the rat following a single intravenous injection of "marker particles Two types of experiments were performed (*a*) the cellular uptake phase was investigated by giving Thorotrast® injections to rats with fractures of different age and fixing the tissue 8–9 hours after the injection, (*b*) the storage capability of the cells was studied by injecting the rats with Thorotrast® at the time when the fracture was made and fixing the tissue at different intervals thereafter

MATERIALS AND METHODS

30 *female Sprague Dawley* rats weighing 150–200 gms, maintained on ordinary solid pellets and tap water *ad lib*, were utilized for the experiments A complete fracture of the shaft of the femur was produced digitally or by means of a rubber dressed hemostat under light ether anesthesia. As a rule *both femurs were fractured* To one half of the rats 0 5 ml Thorotrast® (Testagar, Detroit, Mich. U S A) (25 per cent stock solution) was given intravenously immediately prior to fracturing In the second group the same dose of Thorotrast® was given 8 9 hours before the rats were sacrificed The animals were sacrificed by decapitation—in groups of two—on days 3, 5, 6, 7, 10, 12, 13, 14, 17, 19 25 28, 31, 33, and 34 after the fracture The *fractures were immediately exposed and dissected* free for gross examination, and samples from the fracture sites and calluses were excised with a razor blade both from the fracture gap and the periosteal areas for electron microscopic investigation The excised tissues were carefully trimmed to cubes with a side of ~ 1 mm under a drop of fixative and were subsequently immersed in the same fixative Fixation was performed at 4°C in 2 per cent s-collidine buffered osmium tetroxide (OsO_4) (pH 7 4) for 2 hours The tissues were subsequently dehydrated and embedded in Epon Approximately 1 μ thick Epon sections prepared on glass knives were stained with alkaline toluidine blue for orientation in the blocks Areas of tissue selected for

Siemens Elmiskop I electron microscope

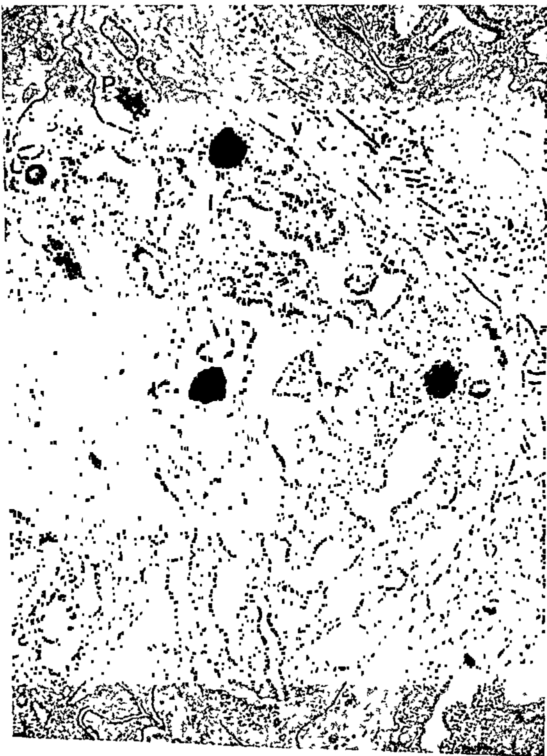
RESULTS

The experiments were well tolerated by all animals Generalized toxic reactions or local cell damage were not observed No harmful effects on the healing of the fractures could be

---

Note All the pictures illustrate the appearance of thin sections of Epon embedded tissues stained with uranyl acetate and lead citrate

Fig 1 Thorotrast® 8 hours, 28 days old fracture. Marker molecules are present in the extracellular space (*arrows*) outside an osteoblast. Thorotrast® macromolecules are also located intracellularly in lysosome like bodies (L) and in a small vesicle (V) immediately beneath the plasma membrane *Note presence* of marker molecules in a pocket (P) formed by the plasma membrane this pocket formation apparently represents an early step in the uptake phase × 30 000


P
V

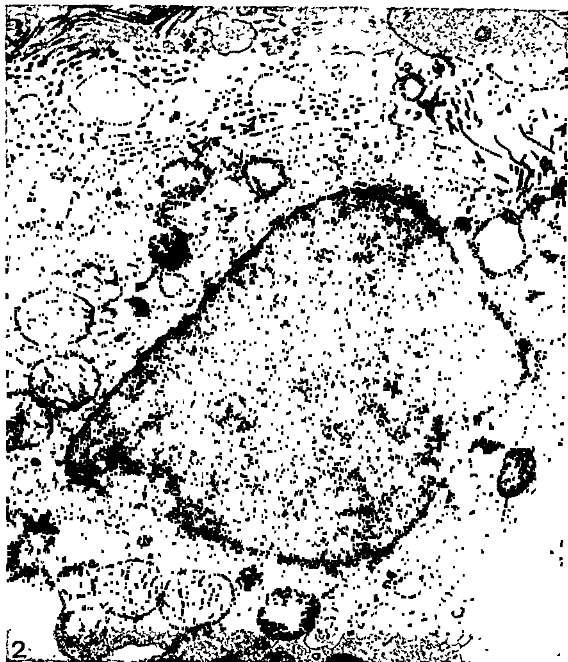


Fig 2 Thorotrast® 8 hours, 28 days old fracture Picture illustrating macrophage-like cell with many vacuoles and dense bodies containing thorium dioxide molecules (*arrows*) × 18,000

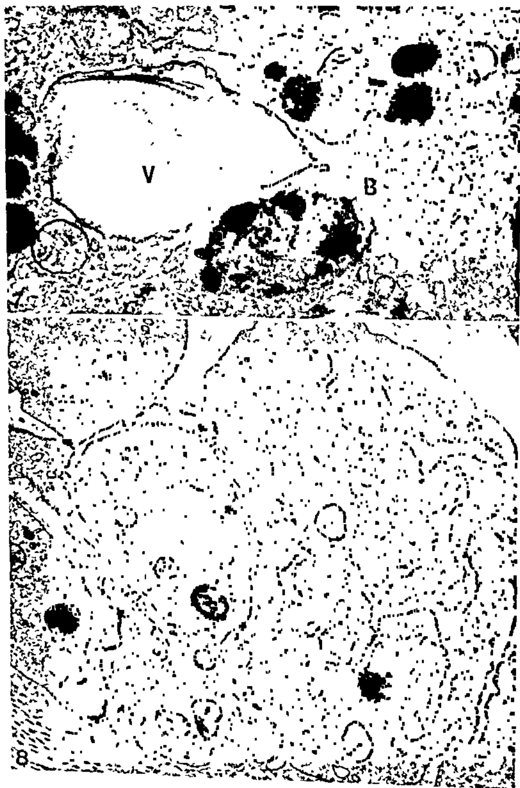
Fig 3 Thorotrast® 8 hours, 28 days old fracture Portion of macrophage-like cell containing large irregulary shaped cytoplasmic bodies filled with marker molecules Possible fusion or fission phenomena are marked by *arrows* × 20,000

Fig 4 Thorotrast® 8 hours, 28 days old fracture Portion of a macrophage like cell with large Golgi apparatus (G) There are several large and small bodies and vesicles located in the vicinity of the Golgi apparatus Note that the small vesicles (*arrows*) are uncoated × 30,000



34*


5


V
B
8

observed as was evident from comparisons with previous morphological studies (11, 12)

In the group of animals where Thorotrast® had been injected 8–9 hours before sacrifice thorium dioxide particles were present both in the extracellular space and within membrane-delimited structures in the cytoplasm of most of the cells in the callus In the group of animals where a longer interval had passed between injection of Thorotrast® and sacrifice of the animals, the marker molecules were no longer to be found in the extracellular space

There are five major types of nucleated cells present in the fracture callus (11) (*a*) cells with apparent predominant phagocytic functions—rich in mitochondria and lysosomes—(macrophages, histiocytes and monocytes), (*b*) osteoclasts characterized by large size, abundance of mitochondria and lysosome-like elements, multiple nuclei, and presence of a ruffled border, (*c*) cells with abundant endoplasmic reticulum (and few lysosomes and mitochondria) known to participate in the elaboration of the extracellular collagenous material (fibroblasts, chondroblasts osteoblasts chondrocytes, osteocytes, and various intermediate forms between these cells), (*d*) endothelial and other vascular cells, and (*e*) immature mesenchymal cells. On the basis of purely morphologic criteria the afore mentioned cell types have been considered to represent two functionally different groups of cells (11) *matrix producing cells* and *cells unassociated with matrix production* (with predominant phagocytic ability) The fine structure, distribution and occurrence at different ages of these cells in the callus has been described in a separate publication (11)

Fig 5 Thorotrast® 8 hours 28 days old fracture Area of macrophage like cell containing a huge body with thorium dioxide molecules × 28 000

Fig 6 Thorotrast® 8 hours 28 days old fracture An area of an osteoclast like macrophage with well developed ruffled border containing two lysosome like bodies (*arrows*) with Thorotrast® particles en closed × 33 000

Fig 7 Thorotrast® 8 hours 28 days old fracture Part of a macrophage like cell A large vacuole (V) contains a bundle of fibers along with Thorotrast® particles Marker molecules are also present in other membrane limited bodies in the cytoplasm one of which (B) also encompasses tightly packed mem branous materials. × 23 000

Fig 8 Thorotrast® 8 hours 28 days old fracture Portions of cells located close to a capillary Thorotrast® has been picked up by the cell and is located in two small membrane limited bodies in the cytoplasm (*arrows*) These cells have an appearance similar to that of Scott s type A cells (II) × 22,000

Observations on Fractures of Different Age 8–9 Hours after Thorotrast® Injection

Uptake of typical 150–300 Å large thorium dioxide macromolecules in the cells of the callus occurred in all nucleated cells at all intervals studied after the fracture was produced The molecules were found in vacuoles and vesicles located close to the plasma membrane of the cells as well as in other structures deeper in the cytoplasm (see below) Occurrence of similar molecules in the extracellular space and in association with invaginations of the plasma membrane (Fig 1) indicated that the uptake occurred through endocytosis and that the membrane associated vesicles and vacuoles containing marker molecules represented endocytic vacuoles ("phagosomes") The pattern of intracellular distribution of [illegible]

ever for any given cell type [illegible] not vary significantly at the different intervals studied For convenience of presentation, the appearance of the cells constituting the five major functional types will be presented in order below

(*a*) Uptake of Thorotrast® particles in *macrophages histiocytes and monocytes* was performed through formation of endocytic vacuoles of variable size (Fig 2) The vacuoles which were up to 1 μ in diameter, had an electron lucent ("empty") matrix lacking inclusions other than the thorium dioxide molecules Many of these vacuoles were densely packed with macromolecules especially those that were located at some distance from the



Fig 9 Thorotrast® 8 hours, 28 days old fracture An osteoblast with small vesicles (v) subjacent to the plasma membranes containing marker molecules Thorotrast® is also present in lysosome like bodies deeper in the cytoplasm × 21,000



Fig 10 Thorotrast® 8 hours 28 days old fracture Part of a rather immature osteogenic cell (preosteoblast?) Four small Thorotrast® filled bodies (*arrows*) are seen in the cytoplasm G Golgi apparatus with associated coated unlabelled vesicles × 24 000
Inset Part of an osteocyte with two Thorotrast® containing cytoplasmic bodies Note calcified matrix (*arrows*) × 2? 000

plasma membrane Thorotrast®-containing vacuoles situated in the immediate vicinity of the plasma membrane usually had fewer and less densely packed particles These observations suggest that the endocytic vacuoles move toward the interior of the cells and that the enclosed molecules become concentrated during this process (presumably through loss of water and solutes)

In addition to Thorotrast® filled vacuoles with "empty" matrix, many cells contained cytoplasmic bodies of highly variable size which had a homogeneous or finely granular matrix Usually these bodies also contained thorium dioxide molecules (Figs 2–6) These bodies might be found anywhere in the cytoplasm but tended to accumulate in the vicinity of the Golgi apparatus Other Thorotrast®-containing bodies also encompassed fibers with the same dimensions as the collagen fibers in the extracellular matrix (Fig 7), however, a clearly identifiable banding pattern was usually absent from the intracellularly located fibers Finally, electron dense macromolecules were also present in lysosome like elements (containing membranous, vesicular and granular material of the same type as in secondary lysosomes) (Figs 5 and 7) Some of these apparent lysosomes were huge (up to 5 μ in diameter) (Fig 5)

Although there were wide individual variations in the number and size of Thorotrast®-containing vacuoles and cytoplasmic bodies among the phagocytic cells, no clear differences in the ability to take up the marker was noted between the three cell types in this group As a rule, in sections encompassing the *main body of the cells, there* were at least *10* membrane limited vacuoles and cytoplasmic bodies containing thorium dioxide particles, and most of these elements were at least as big as the mitochondria In some Thorotrast®-containing macrophages the plasma membrane tended to form folds and fingerlike projections somewhat reminiscent of the *ruffled* border in osteoclasts (Fig 6) These macrophages also contained numerous Thorotrast® filled vacuoles and bodies

In many instances, large, *irregularly shaped* vacuoles with outpouchings of variable size were noted (Fig 3) Such appearances are compatible with fusion between adjacent vacuoles, although the morphology does not exclude fission Occasionally small vesicles containing from 1 to 5 macromolecules were observed (Fig 4) These vesicles were often located close to Thorotrast® containing vacuoles and might serve as vehicles for transport of molecules to the large vacuoles since they were also encountered near the plasma membrane

(*b*) In the case of *osteoclasts,* marker molecules were found in the channels of the ruffled border and in vacuoles and cytoplasmic bodies located below the brush border as described earlier (14)

(*c*) Uptake of thorium dioxide particles in the *matrix producing cells* appeared to be performed through formation of small endocytic vesicles (diameter $\sim$ 0 2 μ) (Fig 9) Such vesicles were present in the peripheral portions of the cells near the plasma membrane Deeper in the cytoplasm occasional larger membrane limited elements (diameter up to 0 8 μ) packed with marker molecules were observed (Figs 9 and 10) The typical particles were also noted in some lysosome like bodies with membrane fragments and

→

Fig 11 Thorotrast® 8 hours, 28 days old fracture Portion of an immature fibroblast like cell with a membrane-limited body (B) containing both Thorotrast® particles and collagen like fibers × 35 000

Fig 12 5 days old fracture Thorotrast® injected at the time of fracture The picture illustrates a superficial portion of a macrophage containing numerous Thorotrast® filled bodies and vesicles. Note presence of marker molecules in an apparent autophagic vacuole containing a partly digested mitochondrion (m) × 24000

Fig 13 6 days old fracture, Thorotrast® injected at the time of fracture Portion of two cells are shown to the left a fibroblast like cell and to the right a macrophage Note the difference in the amount of Thorotrast® picked up by these two cells While there are only two small lysosome-like bodies possible containing marker molecules in the fibroblast (immediately below the nucleus), numerous irregularly shaped large and small Thorotrast®-filled bodies are seen in the macrophage. × 15 000


11
B
12


13


B
11
12

other densities suspended in their matrix In comparison with the cells with major phagocytic function, Thorotrast®-containing structures in the cytoplasm were fewer and usually smaller Particles were never observed in the collagen containing vacuolar structures described by Göthlin & Ericsson (12)

(*d*) In general *vascular endothelial cells* endocytized comparatively sparse amounts of Thorotrast®, and the pattern of uptake was also in other respects similar to that in mature fibroblasts, chondroblasts and osteoblasts At the 8 hour interval tracer molecules were often still present in the lumen of the vessels and were also observed in the process of penetration to the perivascular tissues

(*e*) Uptake in *immature mesenchymal cells* was highly variable. While in some cells there were rather few and small membrane delimited elements containing Thorotrast® particles (Fig 8) other cells contained large and abundant vacuoles, cytoplasmic bodies and lysosome filled with tracer particles Occasionally, vacuoles containing both marker molecules and collagen like fibers were observed (Fig 11)

Observations 24 Hours and Longer after Thorotrast® Injection and Fracture

Thorium dioxide molecules enclosed in single membrane limited cytoplasmic bodies and lysosome like elements were present in all the different cell types that were found to endocytize Thorotrast® at the 8 hour interval The appearance of some cell types at selected brief and long intervals after the injection is illustrated in Figs 12 to 18 It was found that the localization and distribution of the macromolecules was similar to that at the 8 hour interval with two major exceptions thorium dioxide particles were never encountered in an extracellular location, and endocytic vacuoles and vesicles closely associated with the plasma membrane—and containing tracer molecules—were not present Although—especially at late intervals—there were occasional cells devoid of tracer in the plane of the sections, the vast majority of the cells did show presence of the characteristic macromolecules even 34 days after the injection At no interval were there morphologic signs of cellular damage or necrosis, and evidence for release of marker molecules from the cells of the callus tissue was never obtained

Fig 14 28 days old fracture, Thorotrast® injected at the time of fracture Portion of a macrophage with many lysosome like bodies containing marker molecules × 18 000

Fig 15 28 days old fracture, Thorotrast® injected at the time of fracture. Superficial portion of a chondrocyte with lysosome like bodies (L) containing moderate amounts of marker molecules × 22,500

DISCUSSION

Thorotrast® has been used rather extensively as a macromolecular tracer in ultrastructural research Recent observations of several different cell types favors the concept that cellular uptake of this compound is by way of endocytosis, and that the molecules are transferred to lysosomes of variable functional types (8) The observations in the present study support the notion that all the different cell types in the callus handled the macromolecules in a similar fashion It appeared that at 24 hours and later after the injection, most—if not all—of the marker molecules had been transferred to lysosomes, since the macromolecules were present in single membrane limited bodies containing membranous material and various irregular densities

Two principal groups of cells could be discerned as regards the quantitative aspect of the uptake of the macromolecules The first group included the phagocytic cells (monocytes, histiocytes and macrophages) in which the absorption of thorium dioxide molecules was heavy, resulting in a population of very heterogeneous cytoplasmic inclusion bodies In the second group, consisting essentially of collagen manufacturing cells, the uptake was more restricted In this group the molecules were taken into the cells in small endocytic vesicles, which appeared to transfer their con-

tent to the lysosomes where it was retained for considerable periods of time Some immature fibroblasts appeared to belong to the phagocytic group in that they showed a larger uptake than mature fibroblasts chondroblasts and osteoblasts and likewise often appeared to absorb marker molecules into vacuoles of variable size and form Thus in essence the present findings support previous suggestions based on electron microscopic observations concerning the functional potentialities of the phagocytic cells in fracture callus (11) However some immature fibroblasts had a much more developed ability for endocytosis than one could expect from the fine structural appearance alone and the more mature matrix producing cells were also able to ingest considerable amounts of extraneous material

Involvement of macrophages in collagen resorption in other tissues undergoing functional remodeling has been reported for instance during uterine involution (4 22) in macrophages surrounding hair follicles (23) and in the tadpole tail (10 24) Moreover the existence of fibroblasts (4) and fibroblasts changed into phagocytes (21) with a capacity of resorbing and degrading collagen fibers intracellularly during uterine involution has been reported *Knese* (20) in an ultrastructural study of the epiphysis of cattle fetuses and rats suggests that mononucleated cells are of significance in the resorption of intracellular substance including collagen Multinucleated cells and small round cells have been described to play a role in removing collagen (17)

Information obtained in the present study favors the concept that during fracture healing collagenous material in the matrix is ingested by phagocytes including monocytes histiocytes and macrophages The uptake and storage of Thorotrast® particles in these cells was seen to occur in cytoplasmic vacuoles with the ultrastructural characteristics of heterophagic vacuoles and secondary lysosomes in some instances these same structures also contained filamentous collagen like material This suggests that the aforementioned phagocytic cells represent devices for controlled degradation of collagenous and other phagocytized extracellular material This interpretation is supported by recent electron microscopic findings indicating presence of acid phosphatase activity in fiber containing vacuolar structures in the same types of cells (13) It would therefore seem that macrophage like cells—in addition to osteoclasts—have important functions during resorption and remodeling of bone tissue especially at early stages

The finding of collagen like fibers in cytoplasmic vacuoles also containing thorium dioxide molecules and located in central portions of immature fibroblasts suggests that these cells can also resorb collagen There also appeared to be a general—although less conspicuous—ability of all the bone forming cells in the callus to perform endocytosis of macromolecules This suggests that these cells are not only engaged in synthetic tasks but may also have a resorptive function It is interesting to note in this connection that it has been postulated that osteocytes have an osteolytic ability [for a review see *Belanger* (3)] Furthermore *Irving* & *Heeley* (17) and *Irving* & *Bond* (16) have shown that small round cells present in calcified tissues have resorbing potentials and *Baud* (1 2) suggests that osteocytes have resorptive functions in bone tissue

As shown by *Gothlin* & *Ericsson* (12) mature fibroblasts as well as osteoblasts and chondroblasts sometimes contain vacuolar structures with enclosed collagen like material The absence of marker molecules in these structures supports the notion that they represent a type of secretory vacuole encompassing newly synthesized collagen (12)

Fig 16 28 days old fracture Thorotrast® injected at the time of fracture Part of an osteoblast with two Thorotrast® containing lysosome like structure (L) in the cytoplasm G Golgi apparatus with associated coated vesicles (*arrows*) × 22 500

Fig 17 31 days old fracture Thorotrast® injected at the time of fracture A macrophage like cell with many small and large Thorotrast® filled bodies in the cytoplasm × 24 000



17

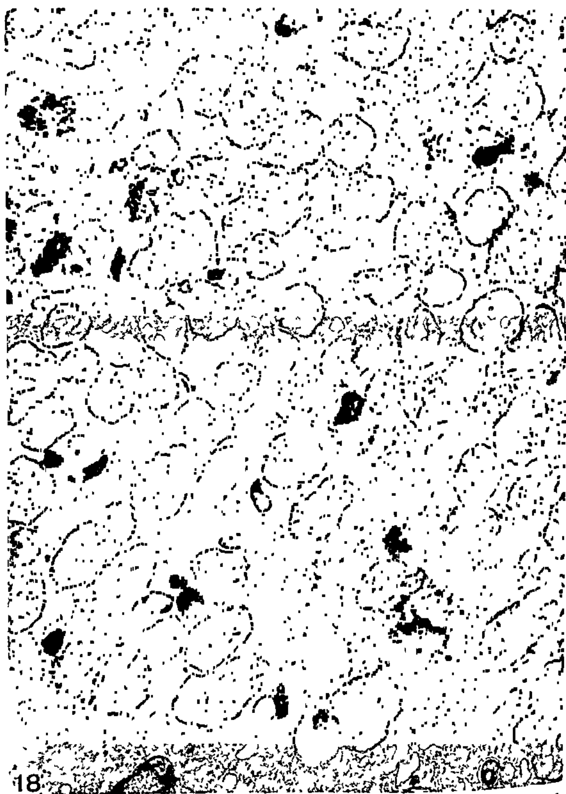


Fig 18 34 days old fracture, Thorotrast® injected at the time of fracture Portion of an osteoclast containing several Thorotrast®-filled bodies (*arrows*) × 20,000

Thorium dioxide particles appeared to be retained for long periods of time in the lysosomes of all the cells present in the callus Since the macromolecules were never encountered in the extracellular space of the callus tissue 24 hours and later after the injections, no or very limited extrusion of lysosomal content appeared to occur Hence, 'reutilization" as an explanation for the intracellular occurrence of marker molecules at late intervals can probably be excluded It would therefore seem that "labelling" of the cells with Thorotrast® might be a useful means of studying the development, fate, and histogenesis of the different types of cells in the callus It would further appear that "secretion" of lysosomal enzyme by way of "reverse endocytosis" is uncommon or nonexisting in the cell types studied Thus, the lysosomes in these cells appear to be stable organelles with a long life span and slow turnover

Vesicles associated with the Golgi apparatus ('Golgi vesicles") are both of the "coated" and "uncoated' variety in the different cells constituting the callus (Figs 4 and 10) (11) Some of these vesicles—in osteoblasts—appear to contain acid phosphatase and may correspond to primary lysosomes (15) The observations in the present study indicate that the Golgi vesicles" may also derive from the plasma membrane and represent endocytotic vesicles It seems likely—when the results of the present study are compared with those of previous investigations (11, 15)—that the coated Golgi associated vesicles are primary lysosomes, while the uncoated ones are of endocytic origin

---

This investigation has been supported by grants from the *Swedish Medical Research Council* (projects B72 12X 1006 07A and B73 12X 1006 08B)

The assistance of Miss *Silvia Mengarelli*, Mrs *Britt Marie Akerman* and Mr *Magnus Norman* is gratefully acknowledged

REFERENCES

1 *Baud C A* The fine structure of normal and parathormone-treated bone cells. In "Proc. Fourth Europ Symp. Calcified Tissues', Leiden 1966 (Gaillard P J, van den Hooff A & Steedijk, R., eds) Int Congress Series No 120, Excerpta Medica Foundation, Amsterdam, pp 4–6

2 *Baud, C A* Submicroscopic structure and functional aspects of the osteocyte Clin Orthop 56 227–236, 1968

3 *Belanger, L F* Osteocytic resorption In "The Biochemistry and Physiology of Bone vol. III, pp 239–270, (Geoffrey H Bourne, ed) Academic Press, New York and London, 1972

4 *Brandes, D* & *Anton, E* Lysosomes in uterine involution Intracytoplasmic degradation of myofilaments and collagen J Gerontology 24 55–69, 1969

5 *Brunk, U* Distribution and shifts of ingested marker particles in residual bodies and other lysosomes Studies on *in vitro* cultivated human glia cells in phase II and III Exptl Cell Res (1973) (in press)

6 *Brunk, U, Ericsson, J L E, Ponten, J* & *Westermark, B* Specialization of cell surfaces in contact inhibited, human glia like cells *in vitro* Exptl Cell Res 67 407–415, 1971

7 *Casley-Smith, J R* & *Reade, P C* An electron microscopic study of the uptake of foreign particles by the livers of foetal and adult rats Brit J Exp Path 46 473–480, 1965.

8 *Daems, W T, Wisse, E* & *Brederoo, P* Electron microscopy of the vacuolar apparatus In Lysosomes in Biology and Pathology', (Dingle, J T & Fell, H B, eds), vol 1, pp 64–114 Amsterdam and London North Holland Publ Company, 1969

9 *Ericsson, J L E* & *Engberg, A* Electron microscopic studies on methods for *in vivo* labelling of lysosomes In 'Electron Microscopy 1968 (Proceedings of the 4th European Regional Conference on Electron Microscopy, Rome, Italy, Sept 1 7) (Bocciarelli, D S, ed) Tipografia Poliglotta Vaticana, Vol 2, pp 239–240 (1968)

10 *Gross, J, Nagai, Y, Lapiere, C H, Usuku, G, Grillo, H C* & *Eisen, A Z* Mechanisms of connective tissue remodelling and resorption during anuran metamorphosis In 'Structure and Function of Connective and Skeletal Tissue' (Tristram, G R, ed), pp 426–430, London, Butterworths, 1965

11 *Gothlin, G* Electron microscopic observations on fracture repair in the rat Acta path microbiol scand Section A 81 507–522, 1973

12 *Gothlin, G* & *Ericsson, J L E* Electron microscopic studies of cytoplasmic filaments and fibers in different cell types of fracture callus in the rat. Virchows Arch Abt B Zellpath 6 24–37, 1970

13 *Gothlin, G* & *Ericsson, J L E* Unpublished observations (1972)

14 *Gothlin, G* & *Ericsson, J L E* Observations on the mode of uptake of thorium dioxide par

ticles by osteoclasts in fracture callus Calc Tiss Res *10* 216–227, 1972

15 *Gothlin, G & Ericsson, J L E* Fine structural localization of acid phosphomonoesterase in the osteoblasts and osteocytes of fracture callus Histochemie (1973) (in press)

16 *Irving, J T & Bond, J A* The subcutaneous implantation of autogenous rat molars Amer J Anat *123* 119–130, 1968

17 *Irving, J T & Heeley, J D* Resorption of bone collagen by multinucleated cells Calc Tiss Res. *6* 254–259, 1970

18 *Kluge, T* Pericardial absorption of thorium dioxide in rats I An electron microscopical study Acta path microbiol scand *72* 64–86, 1968

19 *Kluge, T & Hoeig, T* Ultrastructural localization of Thorotrast® in the reticuloendothelial system Amer J Path *54* 355–371, 1969

20 *Knese, K H* The ultrastructure of resorbing cell borders Calc Tiss Res. *4* 78–79 (suppl), 1970

21 *Luse, S & Hutton, R* An electron microscopic study of the fate of collagen in the post partum rat uterus Anat Rec *148* 308, 1964 (abstract)

22 *Parakkal, P* Involvement of macrophages in collagen resorption J Cell Biol *41* 345–354 1969

23 *Parakkal, P* The hair growth cycle J Cell Biol *47* No 2, part 2, Nov 1970, abstract no 640, p 223a (X Ann Meeting Am Soc. Cell Biol)

24 *Weber, R* Ultrastructural changes in regressing tail muscles of *Xenopus* larvae at metamorphosis *J Cell Biol 22 481–487, 1964*

Acta path microbiol scand Section A 81, 543–551, 1973

GYNECOMASTIA

Enzyme-Histochemical and Histological Investigations with a Correlation of Enzyme Activities in Gynecomastia and Fibro-adenomatosis

HENNING JENSEN

The University of Pathological Anatomy, Rigshospitalet, Copenhagen and The Department of Pathology, Bispebjerg Hospital, Copenhagen

In 20 cases of human gynecomastia the following histochemical reactions were investigated NADH-Tr, NADPH Tr, LDH, SDH, G 6 DH, α GPDH and non specific alkaline and acid phosphatases The enzyme activity was compared with that found in fibroadenomatosis of women Identical results were obtained in the dehydrogenase reactions apart from G 6 DH, which revealed localized enzyme activity in the proliferating epithelium of the ducts in fibroadenomatosis, which contrasted with the negative findings in gynecomastia. The activity of the non specific alkaline phosphatases was found to be increased in the myoepithelial cells in gynecomastia This is presumably due to hormonal influence The pathological changes and the etiological background are discussed

Many investigations into hyperplasia of the male mammary gland are to be found in the literature, and the pathological features of gynecomastia are well established (1) (4) (9) (19) (31).

The pathological characteristics are described in detail by *Karsner* (19) and *Treves* (31), among others Very few enzyme histochemical studies have appeared in the literature (11) (12) (25) and these publications describe only a few enzyme systems investigated in small numbers of biopsies

Various different enzyme systems have been studied in 20 cases of gynecomastia The findings have been correlated both to those in previous studies and to the author's enzyme-histochemical investigations of the female breast (15) (17)

Received 22 xi 72 Accepted 23 ii 73

Requests for reprints should be addressed to Dr *Henning Jensen, Nymindevej 13, Vanløse, 2720 København*

35*

MATERIAL AND METHODS

In a consecutive material consisting of tissue from mammary glands of 500 patients 20 biopsies were from the male breast In all these cases the histological diagnosis was gynecomastia

The tissue was obtained peroperatively and immediately frozen in isopentane cooled by a mixture of acetone and dry ice to about –80°C The frozen tissue was stored at –20° C until used Enzyme histo chemical methods were employed for identification of the following enzyme activities

NADH tetrazolium reductase (NADH-Tr), NADPH tetrazolium reductase (NADPH Tr), lactic acid dehydrogenase (LDH), succinic acid dehydrogenase (SDH), glucose-6-phosphate dehydrogenase (G-6 DH), α glycerophosphate dehydrogenase (α GPDH), non specific alkaline phosphatase (alk ph) and non-specific acid phosphatase (acid ph)

Fresh frozen tissue was cut on a cryostat (SLEE, Pearse) at about 8 microns. Unfixed tissue sections were used in all the enzymatic reactions

All the dehydrogenase tests were carried out as described by *Thomas* & *Pearse* (30), using N BT as indicator The tissue specimens were incubated for 20 and 30 minutes at a temperature of 37° C The method used for detection of acid ph was that described by *Barker* (3), using α naphthyl phosphate as the specific substrate The indicator

was pararosanilin, nuclear stain methyl green Alk ph was demonstrated according to the method described by *Burstone* (7) using naphthol AS BI-phosphate as the specific substrate and Fast Red Violet LB as indicator Hematoxylin was used as nuclear stain The incubation period was 20 and 30 minutes at 20° C for both alk ph and acid ph

Tissue specimens stained by the above methods but without specific substrates, were used as controls.

Corresponding tissue sections stained with hematoxylin-eosin were used in the histological evaluation

In the semiquantitative estimation of the enzyme activity the following grading was employed

0 no reaction
1 slight reaction the structure of the tissue was barely visible
2 slight but definitely positive reaction
3 pronounced and distinct reaction without excessive staining of the tissue
4 blurring of the tissue structure caused by excessive staining due to the high enzyme activity

RESULTS

Histology

In the routine sections stained by hematoxylin eosin there was an increased number of ramified ducts The lumen of the ducts varied in shape and size, and sometimes cystic dilations were present The lumen could appear twisted and occasionally disfigured due to the presence of nodular proliferation of the surrounding connective tissue which caused compression of the lumen The epithelium of the ducts was often found to be moderately proliferative No cell atypia was found in the duct epithelium There was no lobular structure in the glandular tissue Secretion was observed in a few ducts Sometimes the structure of the periductal connective tissue differed from the remaining stroma in such cases there was often edema and some basophilia of the intercellular substance could be found close to the ducts (mucoid degeneration) An inflammatory infiltrate consisting of mononuclear cells, was frequently observed, but rarely marked

Dehydrogenase Enzyme Activity

NADH-Tr showed pronounced activity in the duct epithelium The reaction product (formazan granules) was scattered throughout the cytoplasm of the cells The cell nucleus showed no reaction and appeared as pale circular structure surrounded by the blue stained cytoplasm (Fig 1 and 2) In the individual ducts the intensity was approximately the same from cell to cell, while a moderate variation in intensity was seen when different ducts were compared The average activity was 3 The myoepithelial cells showed the same activity as the remainder of the epithelium

In the surrounding stroma there was activity localized to fibroblasts and capillaries The fibroblasts displayed a slight but distinct activity of grade 1 to 2 intensity, but the activity was variable Commenly the strongest activity was seen in the periductal tissue (Fig 1), but in biopsies where the periductal tissue and the surrounding stroma were of the same morphological configuration the fibroblast activity was identical The activity localized to the capillaries was grade 1 to 2 like that in the fibroblasts, with an even distribution of the reaction product in all capillaries Activity was occasionally observed in the lipid cells In these cases the reaction was localized as a narrow border along the cell membrane while the central area of the cells containing lipid showed no reaction

NADPH-Tr and *LDH* corresponded to NADH Tr both as regards localization and intensity

The *SDH* and *G-6 DH* activity was weak and localized only to the duct epithelium. The maximum activity was *1*, but in many cases the structure showed no reaction Neither fibroblasts nor capillaries showed any reaction (Fig 3)

α GPDH activity was identical to SDH in localization and intensity In the lipid cells an activity of grade *1* was frequently seen this activity was confined to the cell membrane No reaction was seen in fibroblasts or capillaries

Phosphatase Activity

Alkaline phosphatase In the epithelium of the ducts the enzyme activity varied from





Fig 1 NADH Tr Heavy deposit of dye s seen in the epithel um of ducts indicating strong act tity Note activity in the f broblasts in the periductal stroma. Magn fication × 25

Fig 2 NADH Tr Strong activity in duct epithe lium. The stromale f broblasts are fairly reactive Magn f cation × 70

Fig 3 SDH Sl ght activity in duct epithelium No activity in stromal cells Magn f cat on × 70

Fig 4 Alk ph Activity in myoepithelial reg on No reaction in adjacent epithelium of duct Note the react on in capillaries (arrow) Magnif cat on × 70

Fig 5 Alk. ph Intense activity is seen in myo epithel al cells The thick layer of proliferating epi thelium is unreactive Note the reaction in cap l laries (arrows) No activity in stromal cells Magni f cation × 150

Fig 6 Ac d ph The activ ty is demonstrated in the luminal ep thelium. No activity in myoep the- l al reg on and stromal cells. Magn f cat on × 50

TABLE 1 *The En..*

	Epithelium							
Age	NADH-Tr	NADPH-Tr	LDH	SDH	G-6 DH	α GPDH	Alk ph	Acid ph
37	3	–	3	1	1	1	1–2	0
36	3	3	2	0	0	0	2	1-2
53	2	2	2	0–1	0–1	1	0	0
71	3	3	3	0	0	1	0	1 2
22	3	3	2	1	1	1–2	0	0–1
28	2–3	2	2–3	0	0	0	0	1 2
35	3	2	3	0–1	0–1	0–1	0	0–1
50	2–3	2–3	2–3	0	0	0	0	0–1
56	3	3	3	0	0	0–1	0–1	0–1
24	2–3	2–3	2	0–1	0	0–1	0	0–1
79	3	3	3	0	0	0–1	0	0–1
24	3	2	3	0–1	1	0–1	0–1	0 1
28	3	2–3	3	0	0	0	0	0
63	3	2	3	0	0	0	0	0
29	3	2–3	2–3	1	0	1	0	0
22	2–3	2 3	2–3	1	0–1	1	0	1
65	3	3	3	0–1	0–1	0–1	1	1 ?
25	2–3	2	2	1	0	0–1	0	0–1
22	3	3	2	0–1	0–1	0–1	0	0–1
81	3	2–3	2	0	0	0	0	1 2

In the left side of the table the activity of 8 enzyme systems in the duct epithelium is shown, while the right half of the table shows the corresponding enzyme activity in the fibroblasts The column on the far right shows the activity of non specific alkaline phosphatase in the myoepithelial cells

TABLE 2 *The Average Activity of 8 Enzy...*

Epithelium							
NADH-Tr	NADPH Tr	LDH	SDH	G 6 DH	α GPDH	Alk ph	Acid ph
3	2–3	2–3	0–1	0–1	0–1	0	1

The left half of the table shows the activity in the duct epithelium, while the fibroblast activity is shown in the right half of the table The column on the far right shows the activity of non specific alkaline phosphatase in the myoepithelial cells

areas without activity to activity of grade 3 This variation was found even within the same biopsy However, the activity of the luminal epithelial cells was consistently lower than that of the myoepithelial cells These latter cells demonstrated an activity of grade 3 to 4, and thus dominated the picture in the alk ph reactions (Fig 4 and 5)

The alk ph activity of the fibroblasts varied considerably This was also true of cells from the same tissue specimens *In some* cases (case no 12, 15, 17 and 20) groups of

ivity in 20 Cases of Gynecomastia

NADH Tr	NADPH Tr	LDH	SDH	G 6 DH	α GPDH	Alk ph	Acid ph	Alk ph in myoepithelial cells
Fibroblasts								
1–2	1	1–2	0	0	0	1	0	3
1	1	1	0	0	0	0	0	3
1	1	1	0	0	0	0	0	4
2	2	2	0	0	0	3	0	3
1	1	1	0	0	0	0	0	3
1	1	1	0	0	0	0	0	3
1 2	–	1–2	0	0	0	1 2	0	3–4
0–1	1	0–1	0	0	0	0	0	3
1	1	1	0	0	0	0	0	4
1	1	1	0	0	0	1	0	3
1 2	2	1 2	0	0	0	2	0	3–4
1–2	1 2	1–2	0	0	0	3	0	4
1	1	1	0	0	0	0	0	0
1–2	1	1–2	0	0	0	1–2	0	3
1 2	1	1	0	0	0	2	0	4
1–2	1–2	1	0	0	0	0	0	4
1–2	1–2	1–2	0	0	0	3	0	4
1	1	1	0	0	0	1–2	0	4
1	1	1	0	0	0	0	0	3
2	1	1	0	0	0	1–2	0	4

Systems in 98 Cases of Fibroadenomatosis

NADH Tr	NADPH Tr	LDH	SDH	G 6 DH	α GPDH	Alk ph	Acid ph	Alk ph in myoepithelial cells
Fibroblasts								
1 2	1	1	0	0	0	1	0–1	2–3

fibroblasts with enhanced alk ph activity were found around the ducts

Marked enzyme activity (grade 3) was consistently found in the capillaries (Fig 5)

Acid phosphatase There was a weak enzyme activity in the epithelium The formazan granules were confined to the luminal part of the cells in those biopsies in which the reaction was positive (Fig 6) In general the intensity of the reaction was grade 0 to 1 In a few ducts the lumen contained secretion in which an activity of grade 2 was

demonstrated In most cases the myoepithelial cells showed no enzyme activity, but in a few cases grade 1 activity was seen As the same cases were characterized by a high activity of alk ph, the weak activity of acid ph could well be due to non specific staining No acid ph activity was found in the fibroblasts or capillaries

DISCUSSION

Comprehensive descriptions of the pathological changes in gynecomastia have been published by, for example, *Karsner* (19) From the literature and his own observations *Karsner* has given the following *definition of gynecomastia*

'Gynecomastia is an enlargement of the mammary gland and glands of males due to proliferation of connective tissue, dense in the general stroma and often loosely arranged in periductal regions, together with variable degrees of multiplication, elongation, or branching of ducts, or all three, without formation of true acini, accompanied by periductal or more widespread infiltration of lymfocytes plasma cells, large mononuclear cells, and occasionally eosinophils or neutrophilic polymorph nuclear cells, or both, secretion is frequently present in ducts may be discharged spontaneously or manually expressed but rarely if ever is it true colostrum or milk

This definition excludes pseudogynecomastia due to the deposition of fat in the mammary gland as well as suppurative or other essentially inflammatory processes, granulomatous lessions and neoplasms either benign or malignant '

This description includes all the characteristic morphological elements since discussed in the literature

Treves (31) investigated 476 cases of gynecomastia His findings were in accordance with *Karsner*s definition which is still valid (1) (9) (14)

Where the etiological background of gynecomastia is concerned a large number of cases must be regarded as secondary to hormonal disturbance due to pathological changes in other organs (14) Tumours of the testis (8) (31) and cortical tumours of the adrenals (29) (32) are examples of tumours causing this secondary form of gynecomastia Atrophy of the testis caused by trauma or infection (2), hyperthyroidism (5) (21), cirrhosis of the liver (4) (23), and adenoma of the pituitary gland (24) are other disorders which may have the same effect In some cases the gynecomastia seems to be caused by drugs e g digitalis (6) (22) (28)

Among the 20 patients in the present study the Klinefelter syndrome was found in one case (case no 13) (Table 1)

It is well known that gynecomastia is a frequent complication to this syndrome (20) In case no 19 the patient was operated on for a retention of the testis and in this case the gynecomastia may be regarded as secondary to trauma and atrophy of the testis (2) In case no 9 and case no 14 diabetes mellitus and chronic pancreatitis, respectively were present In both cases there was a history of moderate severe chronic alcoholism but no cirrhosis of the liver was demonstrated In case no 8 there was a carcinoma of the thyroid gland, but no hyperthyroidism was observed and no medical treatment had been administered prior to operation

In case no 12 and case no 17 digitalis was administered for 8 years and 9 months respectively before they came to operation In these cases digitalis may well have been the causative factor in the gynecomastia (6) (22) (28)

Case no 20 suffered from epilepsy and barbiturates mysoline and diphenylhydantoin had been administered for a number of years before the biopsy was taken There is no description in the literature of these drugs acting as causative agent in gynecomastia

In the remaining 12 cases the gynecomastia was not accompained by any other signs of disease and in these cases the gynecomastia must therefore be classified as idiopathic

Table 1 contains the semiquantitative values of the enzyme activities investigated The values in the duct epithelium and fibroblasts

are shown In addition, the activity of the alk ph in the myoepithelial cells is shown separately

Table 2 contains values from corresponding enzyme studies in human fibroadenomatosis *The average values for 98 cases are given* in the Table (16) It must be emphasized that in the present study no tissue specimens from normal male mammary gland were avaieable as reference values for the enzyme activities found in gynecomastia The same was true of the investigation of fibroadeno matosis in women The necessity of employing suitable tissue and tissue preparation in order to ensure truly normal reference material has frequently been neglected in the literature For example, the following material has often been used

1) autopsi material or 2) tissue from mammary glands in which there was a localized *pathological process (benign or malignant)* These must be rejected as being unsuitable for reference purposes on the following grounds

1 Autopsy material should be avoided owing to possible *post mortem* enzyme changes
2 Where there is a localized pathological process in the breast it is conceivable that the factors which have given rise to the pathological process may also affect the enzyme activity in the neighbouring tissue

Because of this we have decided to compare the alterations in enzyme activity in gyneco mastia with those in fibroadenomatosis in women

Comparison of Tables 1 and 2 reveals that the enzyme activity is almost identical in gynecomastia and fibroadenomatosis

In both conditions NADH-Tr, NADPH Tr and LDH were the most active while there was only weak activity of SDH, G 6 DH and α-GPDH

According to *Mori et al* (25) the activity of G 6 DH in gynecomastia is relatively low, this is confirmed in the present study The activity of G 6-DH was usually equally low in fibroadenomatosis, but here there was a greater variation between the biopsies, with pronounced enzyme activity in the proliferat ng epithelium in some of the ducts The presence of apocrine epithelium in the ducts often led to increased activity of this enzyme As only the averages of the enzyme activities found in fibroadenomatosis are given in Table 2, this variation in enzyme activity is not readily apparent from the figures

In gynecomastia the proliferating epithelium of the ducts showed no increase in G-6 DH, in contrast to the findings in fibroadeno matosis

In the myoepithelial cells the activity of the non specific alk ph differed in gynecomastia and fibroadenomatosis In all but one of the cases of gynecomastia this enzyme ac tivity was marked In the last case (no 13) no enzyme activity could be demonstrated As the enzyme activity of the capillaries in this biopsy was equally low, it is possible that there may have been a technical error In fibroadenomatosis the activity of alk ph in the myoepithelial cells varied considerably in the individual tissue specimens, as well as from one biopsy to the next. This is not ap parent from Table 2 due to the use of average values in the table In the fibroadenomatosis material the activities found in the myoepithelial cells were

0 6 cases 0–1 4 cases, 1 5 cases, 1–2 4 cases, 2 15 cases 2–3 6 cases, 3 29 cases 3–4 14 cases 4 14 cases

This variation in the values in fibroadeno matosis contrasted with the uniform findings in gynecomastia (Table 1) In the literature it is repeatedly stated that there is an decrease in alk ph in the myoepithelial cells after the menopause (10 (11) (13) (26) (27) It has been suggested that this is secondary to a decrease in estrogen activity on the breast tissue The high activity found in the myoepithelial cells may be explained as being due to increased estrogen influence on the male breast tissue *Karsner* (19) has suggested that gynecomastia may be secondary to hyperestrogenism and *Karnauchow* (18) stated that gynecomastia was characterized by

hyperplasia of the myepithelial cells. In the capillaries there was always marked alk ph activity The alk ph reaction may be used for evaluation of the structure of the capillaries, due to the red staining of the capillaries wall This method has previously been used by *Fanger & Barker* (12) for the evaluation of the structure of capillaries in breast tissue These authors reported a decrease in the number of capillaries in gynecomastia This finding has not been conformed in the present study, in which the number of capillaries in gynecomastia and fibroadenomatosis was found to be identical No difference in alk ph activity of the fibroblasts was found when the gynecomastia and fibroadenomatosis specimens were compared The percentage of cases in which groups of fibroblasts with increased alk ph were seen around the ducts seemed to be comparable in the two disorders

It would seem that the alk ph activity of the fibroblasts was unaffected by hormones, Thus there was no variation in activity with age, similarly no relationship between hormone treatment and alk ph activity of the fibroblasts has been recorded in previous investigations of fibroadenomatosis (15) (17)

CONCLUSION

Identical activity of the enzymes NADH-Tr NADPH-Tr LDH SDH, α GPDH and acid ph has been found in breast tissue from cases of gynecomastia and fibroadenomatosis

G 6 DH activity was weak in both fibroadenomatosis and gynecomastia, but in fibroadenomatosis there was often a localized increase in the proliferating epithelium of the ducts The alk ph activity was found to be increased in gynecomastia as compared with fibroadenomatosis, possibly due to hormonale influence

The work was aided by grants from *Doell Fonden* and *P Carl Petersens Fond*

REFERENCES

1 *Anderson, W A D* Pathology 6 ed vol 2 C W Mosby Co, St. Louis, 1971 p 1580-1582

2 *Apert, E & Declèty, J* Gynecomastie unilatérale á la suite de traumatisme des bourses. Bull et mem Soc med hop Paris 42 1091 1095, 1918

3 *Barka, T & Anderson, P J* Histochemistry Hoeber, New York, London 1963, p 242 244

4 *Bennett, H S, Baggenstoss, A H & Butt, H R* The testis, breast, and prostate of men who die of cirrhosis of the liver Am J Clin Path. *20* 814–828, 1950

5 *Berson, S A & Schreiber, S S* Gynecomastia and hyperthyroidism J Clin Endocrinol. 13 1126–1128, 1953

6 *Bloch, K* On the pathogenesis of breast hypertrophy from digitalis therapy Ztschr Kreislaufforsch *50* 591–595 *1961*

7 *Burstone, M S* Histochemical comparison of naphthol AS phosphates for the demonstration of phosphatases J Nat Cancer Inst *20* 600-612, 1958

8 *Daly, D W, Dossett, J A & Jull, J W* An oestrogen secreting sex-chromatin positive teratoma of the testis associated with gynecomastia Brit J Surg *50* 816–819, 1963

9 *Duitt, R W, Stewart F W & Berg J W* Tumors of the breast Atlas of Tumor Pathology, Second Series, Fascicle 2 Armed Forces Institute of Pathology Washington DC 1968

10 *Drennan, J M* Alkaline phosphatase in breast carcinomas Brit J of Surg 39 458-461 1951 52

11 *Fanger H & Barker, B* Histochemistry of the breast disease Arch Path *67* 293–305 1959

12 *Fanger H & Barker, B* Capillaries of normal and diseased breast Arch Path *69* 67 76 1960

13 *Huseby A R & Thomas L B* Histological and histochemical alterations in the normal breast tissues of patients with advanced breast cancer being treated with estrogenic hormones. Cancer 7 54–74 1954

14 *Haagensen C D* Diseases of the breast 2 ed W B Saunders Co London 1971 p 76 87

15 *Jensen H* Fibroadenomatosis and breast carcinoma Acta Path microbiol scand Sect A *78* 421-431 1970

16 *Jensen H* Enzymhistokemiske undersøgelser

17

ated by the aid of the alkaline phosphatase

activity Acta Path microbiol Scan Sect A *79* 321–329, 1971

18 *Karnauchow, P N* Myo-epithelium in gyne comastia. Amer J Path *30* 1169 1179, 1954

19 *Karsner, H T* Gynecomastia Amer J Path *22* 235 315, 1946

20 *Klinefelter, H F, Reifenstein, E C & Al bright, F* Syndrome characterized by gyneco mastia, a spermatogenesis without a leydigism, and increased excretion of follicle stimulating hormone J Clin Endocrinol *2* 615–627, 1942

21 *Larsson, O, Sundbom, C M & Åsted, B* Gynoecomastia and diseases of the thyroid Acta Endocrinol *44* 133–137, 1963

22 *LeWinn, E B* Gynecomastia during digitalis therapy, report of 8 additional cases with liver function studies. New England J Med *248* 316–320, 1953

23 *Lloyd, C W & Williams, R H* Endocrine changes associated with Laennec's cirrhosis of liver Am J Med *4* 315–330, 1948

24 *McCullagh, E P & Rossmiller, H R.* Methyl testosterone I Androgenic effects and the production of gynecomastia and oligospermia J Clin Endocrinol *1* 496–502, 1941

25 *Mori, M, Sugimara, M, Matsumara, T & Kawashima, H* Histochemical study of the localization of glucose-6 phosphate dehydrogenase in human tumours Gann *54* 433–442, 1963

26 *Murata, I, Hayashi, Y, Muraoka, Y, Miyaji, T & Mori, M* Histochemical studies on hydrolytic enzyme activities in spontaneous mammary cancer of C3H mouse Cancer Res *23* 1665–1668, 1963

27 *Pakdaman, P & Stein, I A* Enzyme histo chemical studies on surgically resected breast Arch of Surg *86* 593–595, 1963

28 *Rodstein, M* Gynecomastia–an unusual mani festation of digitalis toxocity G P *26* 95–97, 1962

29 *Stewart, W K, Fleming, L W & Woltz, H W* The feminizing syndrome in male subjects with adrenocortical neoplasms Am. J Med *37* 455–457, 1964

30 *Thomas, E & Pearse, A G E* The fine localization of dehydrogenases in the nervous system Histochemie *2* 266–282, 1961

31 *Treves, N* Gynecomastia. Cancer *2* 1083–1102, 1958

32 *Wallach, S, Brown, H, Englert, E & Eik nes, K* Adrenocortical carcinoma with gynecoma stia, case report and review of literature J Clin Endocrinol *17* 945–958, 1957

Acta path microbiol scand Section A 81, 552–558, 1973

AN *IN VITRO* STUDY OF THE EFFECT OF CYTOSTATIC DRUGS ON DNA SYNTHESIS IN METHYLCHOLANTHRENE INDUCED MOUSE SARCOMAS AND IN RAT WALKER 256 TUMOURS

LEIF HÅKANSSON and CLAES TROPÉ

Department of Anatomy and Tornblad Institute, University of Lund, Sweden

In the present paper, an *in vitro* test model was used to study the effect of three cytostatic drugs on the DNA synthesis of experimental tumours methylcholanthrene induced primary mouse sarcomas, transplanted methylcholanthrene induced sarcomas (tenth generation), and Walker 256 rat adenocarcinoma. A suspension of single cells and small tissue fragments was prepared and incubated with the following drugs melphalan, vinblastine sulphate, and cytosine arabinoside The effects of the three drugs were measured as the differences in incorporation of tritiated thymidine in drug containing tubes and control tubes The effect of the drugs on the methylcholanthrene induced primary sarcomas showed a great variation which reasonably is not due to technical error as it is markedly reduced if serially transplanted sarcomas or rat Walker tumours were studied A correlation of the effects between melphalan and cytosine arabinoside was observed for the sarcomas

Many efforts have been made to construct an *in vitro* test by which to predict the sensivity of a specific tumour to various cytostatic drugs *Tanneberger* (6) has reviewed the pertinent literature Various methods have been used to keep tumour cells *in vitro* and the effects of the tested cytostatic drugs have been evaluated in various ways In earlier works, the effects on cells were often judged by cytological criteria of cell death More sensitive and also more easy, quantitative methods can be obtained by studies of tumour cell metabolism *in vitro*, especially by the use of radioactively labelled precursors As one of the most imporant aspects of tumour cell metabolism is that associated with cell multiplication, the use of DNA precursors has been utilized by many investigators since *Wolberg* & *Brown* (7) introduced this precursor into the field of cytostatic drug testing

In the present study, we describe such a test system resembling that described by *Mattern et al* (4) Results of two types of experimental tumours are presented mouse sarcomas induced by methylcholanthrene and the transplantable rat adenocarcinoma Walker 256

MATERIAL AND METHODS

Tumour Material

Walker 256 rat tumours were kindly provided by AB Leo (Helsingborg Sweden) Tumours were used for *in vitro* tests nine days after transplantation

Received 30 i 73 Accepted 26 ii 73

Requests for reprints should be addressed to Dr *Claes Tropé*, Tornblad Institute Biskopsgatan 7, S 223 62 Lund, Sweden

Mouse sarcomas were induced in inbred female C57 black mice (Bomholtgard, Ry, Denmark) by subcutaneous injection of 50 μg of 3-methylcholanthrene in 0 1 ml of sesame oil Histologically uniform fibrosarcomas developed appr 3 months after the injection in about 80 per cent of the animals and could be used for the in vitro tests two or three weeks later

In one experiment, a primary sarcoma was serially transplanted for ten generations Transplantation was undertaken by subcutaneous injection of a tumour suspension containing 5 × 10^6 cells. The tumour developing in the tenth generation was further transplanted to ten recipients Three weeks later, these tumours could be used for in vitro tests

In vitro method Trimmed tumour material was cut with scissors in Parker 199 (SBL, Stockholm) and brought into a suspension with small tissue fragments according to the method described by *Borell* (2) Check of cell viability by dye exclusion test showed 10 20 per cent of dead (stained) cells. The cells were washed once by centrifugation with Parker 199, then resuspended in a suitable cell concentration and finally distributed into a number of test tubes The cells were spun down and resuspended in Parker 199 containing the cytostatic drug to be tested Control tubes contained only Parker 199 without cytostatic drugs Three cytostatic drugs were used Melphalan (Alkeran®, Burroughs and Wellcome London) was used in a final concentration of 200 μg/ml vinblastine sulphate (Velbe®, Lilly, Indianapolis) in a concentration of 100 μg/ml, and cytosine arabinoside (Sigma, St Louis) in a concentration of 250 μg/ml Melphalan solutions were always freshly prepared just before the experiment, the other drugs were diluted from stock solutions kept frozen Incubation of cell suspensions with the drugs was performed for three hours Tritiated thymidine (^{3}H TdR, methyl H^3 thymidine, Schwartz/Mann, Orangeburg) with a specific activity of 1 9 Ci/mM was added to a final concentration of 2 μCi/ml and incubation was continued for another hour Within each experimental group, triplet tubes were prepared

The concentrations of cytostatic drugs used were chosen from experiments on normal thymocytes from mice (*Håkansson*, unpublished) We used the lowest concentrations that, in those experiments gave an almost complete inhibition of thymidine incorporation They were tested in a small pilot series of sarcomas and were found suitable

Determination of DNA and radioactivity At the end of the incubation period, the cells were washed once in Parker 199, extracted in 5 per cent trichloroacetic acid (TCA) for 30 minutes at 4° C, washed once in 5 per cent TCA, and finally washed once in absolute ethanol The tubes were left to dry and stored at −20° C until further processed The cell material was subsequently dissolved in 0 6 ml or 1 0 ml of 1 N sodium hydroxide at 37° C for 60 minutes. The DNA content of the sample was determined according to the indole method of *Cerotti* (3) as modified by *Bonting & Jones* (1) The optical density was measured in a Zeiss Spectral Photometer PMQ II at 490 mμ At each series of determinations, a standard curve was run of five double samples using calf thymus DNA The angle coefficient of the standard curve was calculated, and the amount of DNA of the test solutions was expressed as the extinction value read for the test solution divided by the angle coefficient This value is called the adjusted DNA value

0 1 ml of the cells dissolved in sodium hydroxide solution was dissolved in 1 ml of Soluene (Packard Ltd, Stockholm) and 14 ml of scintillation fluid (300 mg dimethyl POPOP, 5 g PPO in 1000 ml Toluene) Radioactivity of the sample was assayed in a Packard Tri Carb spectrometer 3310 during 10 minutes counting The efficiency of this type of preparation was continuously checked by external standardization

Statistical Analysis

The thymidine uptake in a cell sample is determined by the following expression which gives near normally distributed variates (cf *Nordqvist* 5)

$$a = 100 \times \log_{10} \frac{\text{c.p m} \times 10^4}{(\text{ext st}) \times (\text{DNA})}$$

where (ext st) represents the c p m registered with the external standard, and (DNA) the mean of the two adjusted DNA values as determined above The effect of a certain cytostatic drug in a specific experiment is evaluated as the difference between the mean *a* value of the three control tubes and the mean *a* value of the three tubes to which the cytostatic drug was added

RESULTS

Fig 1 shows the effects of the three cytostatic drugs on the ^{3}H TdR incorporation in cell suspensions The ordinate gives the effects as defined above—difference between mean *a*-value in control tubes and the mean *a*-value in tubes containing the cytostatic drug under study In order to evaluate the statistical significance of these differences their errors were calculated as follows

The dispersion of the *a* variates within identically treated tubes (containing the same cell suspension and the same drug) was estimated from all triplet determinations and was

TABLE 1. *Analysis of Interaction between Two So*

Group	df	vinblastine sulphate variance	F
Primary methylcholanthrene induced mouse sarcomas	26	1,659 11	16 83***
Serially transplanted mouse sarcomas	9	565 5	5 73***
Rat Walker 256 adenocarcinomas	9	564 63	11 95***

Ratios (F-values) between variances due to the above-mentioned interaction and the corresponding error variance are given Common error variance for mouse sarcomas is 98 55 at 278 d f, error variance for rat adenocarcinomas is 47 23 at 77 d f *** = p<0 001.

TABLE 2. *Analysis of Variance between Effect Value*

Group	df	vinblastine sulphate variance	F
primary sarcomas	27	1,537 75	4 81**
transplanted sarcomas	10	320	

Variance ratios (F-values) compare the two variances for each drug
** = 0 01>p>0 001, *** = p<0,001

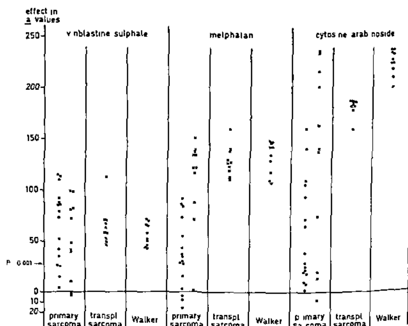


Fig 1. Diagram showing effect values i e differences in *a* values for control tubes and tubes containing the drug to be tested Three groups of tumours primary methylcholanthrene induced sarcomas in the mouse, serially transplanted sarcomas, rat Walker 256 adenocarcinomas *Ten of the primary sarcomas were tested parallel to the one serially transplanted, they are marked with × and are to the right of the other primary sarcomas*

Variation Effect of Cytostatic Drug' and "Tumour"

df	melphalan variance	F	df	cytosine arabinoside variance	F
26	4,302 69	43 66***	26	9,079 41	92 13***
9	137 21	1 39NS	9	67 6	<1 NS
9	824 44	17 46***	9	796 4	16 86***

rimary Mouse Sarcomas and Serially Transplanted Sarcomas

df	melphalan variance	F	df	cytosine arabinoside variance	F
27	4 337 35	22 50***	27	5,994 03	84 45***
10	192 74		10	70 98	

found to be 9 93, based on 278 d f Bartlett's test was applied to see whether different tumours ($\chi^2 = 45\ 79$, 36 d f, NS) or different drugs ($\chi^2 = 5\ 78$, 3 d f, NS) varied with respect to this dispersion, this was not the case The error of the mean difference between two sets of observations, each based on three identically performed tests, is thus

$$9\ 93\sqrt{\frac{1}{3} + \frac{1}{3}} = 8\ 1$$

The 1 per cent significance level is thus a mean difference of 21 and the 0 1 per cent significance level is 27

Responses of different sarcomas show a large variation Using vinblastine sulphate, it varies from 115 to -6, which means that the ^{3}H-TdR uptake in the former tumour, was reduced to appr 7 per cent, being slightly (but not significantly) increased in the latter as compared with the control if tested In order to make certain that this large variation in registered effect is not due to random effects of the technical error, a variance analysis of each of the three materials (one for each drug) was performed, the results are shown in Table 1 It applies to all three cytostatic drugs that there is a very strong and highly significant interaction between the two sources of variation "tumour" and 'response to cytostatic drug" Thus, variations in response of the different tumours are greater than those otherwise expected to be caused by random effects of the technical error It is worth noting that two tumours are resistant towards two of the three tested drugs, and that two tumours are resistent to all three drugs

The differences in registered effects, however, can either represent true differences in the tumours tested or they can be due to *variations in experimental set up from one* day to another Technical variations in the handling of the tumour suspensions etc could give rise to a source of variance which would not be estimated by the triple determinations used for analysis of the technical error This problem is difficult to attack directly, as the same tumour cannot be studied more than

TABLE 3 *Correlation between Effects of the Three Cytostatic Drugs*

Cytostatic drugs compared	Partial correlation coefficient	t value	P
vinblastine sulphate melphalan	0 01	0 07	NS
vinblastine sulphate cytosine arabinoside	-0 17	-0 82	NS
melphalan-cytosine arabinoside	0 69	4 57	P<0 001

Calculated as partial correlation coefficients between mean ^{3}H-TdR incorporation in tubes containing the cytostatic drugs, eliminating incorporation in control tubes
t test of coefficients $\neq 0$

once Instead, we have performed experiments with two sets of experimental tumours which should reasonably be more homogeneous than the primary sarcomas so far discussed

In one of these experiments, tumours were obtained in ten mice by propagation of a sarcoma which, prior to the propagation, had been serially transplanted for ten generations The results of the tests with three cytostatic drugs, performed in a way identical to tests on primary sarcomas, are also given in Figure 1 A variance analysis of interaction between "tumour" and "effect" similar to that used for the primary sarcoma material, was made the results are given in Table 1 Now, no interaction is found for melphalan or for cytosine arabinoside, but there is still a significant interaction for vinblastine sulphate The origin of this interaction (as can be seen in Figure 1) is found in one of the tumours that shows a very strong effect (113) whereas the other nine scatter around 45-75

If a similar experiment was performed with the transplantable Walker 256 rat tumour, all ten tumours proved highly sensitive to the three drugs but an analysis of interaction showed that the tumours actually respond differently to each drug (Table 1) The degree of scatter of the effect values of the ten Walker tumours is obviously of the same magnitude as that of the ten transplanted mouse sarcomas, but the technical error of the former tumours is, for some reason much less than that of the sarcomas Probably there is a residual heterogeneity with respect to effect in the in vitro tests both in the rat Walker tumours and in the serially transplanted mouse sarcomas The possibility that this residual heterogeneity is completely or partly due to technical variations between different experimental occasions cannot be excluded

The scatter of the effect values of the primary mouse sarcomas is obviously larger than that of the values of the serially transplanted sarcomas Table 2 gives the variances applying to the samples of each cytostatic drug and the variance ratios which compare them Significant differences in variances occur in all three groups Thus, the registered differences in response of the primary tumours cannot be a result of technical differences between experiments of the type discussed in the last paragraph It should be noted that ten of the primary tumours were tested in experiments running completely parallel with those in which transplanted tumours were tested These ten primary sarcomas are marked separately in Figure 1, they do not deviate markedly from the remaining 17 sarcomas of this group except for a possibly higher sensitivity to melphalan

Each tumour was tested simultaneously with all three cytostatic drugs, utilizing the same control group In order to explore the possible correlation in response to the three drugs, their effects were compared pair wise using a test for partial correlation

The three variates determined for each tu

mour—namely, the *a* values in control tubes, in tubes containing cytostatic drug 1, and in tubes containing cytostatic drug 2—will be called x_k, x_1, and x_2, resp We now want to determine the correlation coefficient between x_1 and x_2, x_k being eliminated, in other words, we want to study whether the effects of the two drugs somehow covary irrespective of the variation in control tubes We therefore determine the partial correlation coefficient $r_{12.k}$ as

$$r_{12.k} = \frac{r_{12} - r_{1k} \times r_{2k}}{\sqrt{(1 - r_{1k})(1 - r_{2k})}}$$

where the indices to the correlation coefficients mark the variates upon which they are determined

Table 3 gives the result of the partial correlation coefficients Melphalan and cytosine arabinoside show a highly significant covariation—neither co-vary with vinblastine sulphate

DISCUSSION

*The in vitro method chosen in this study re*gisters effects on the thymidine incorporation into DNA Three different drugs were chosen in this study Both melphalan and cytosine arabinoside interfere with the DNA synthesis, the mechanism by which vinblastine sulphate acts is less clear Probably, its interference with DNA synthesis may be secondary to an inhibition of some other cellular metabolic process

Only one concentration level was used for each drug It is possible that finer nuances in response to the drugs could have been obtained by testing the cells with many different concentrations of each drug, but the technical complications in each experiment would also have increased If a too low concentration were used many or even all tumours would appear to be resistant to the drug, if a too high concentration were used, all would appear to be sensitive The concentrations used have resulted in a wide distribution of effects among the primary tumours They therefore seem suitable for the purpose of the study to differentiate between drug sensitivity in different tumours

If the effects of the three drugs were compared, melphalan and cytosine arabinoside were found to show a significant co-variance These drugs act in quite different ways The correlation might be related to a common mechanism in development of drug resistance In this respect, vinblastine sulphate behaved differently from the other two drugs

The effects of the various cytostatic drugs showed a wide dispersion if primary tumours were tested If transplanted sarcomas were tested, this dispersion was markedly and significantly reduced The differences in dispersion is probably due to a better homogeneity of the transplanted tumours The dispersion of the effects in the latter group of tumours shows that the reproducibility obtained by the present method is fairly good Thus, the difference in cytostatic effects on primary sarcomas is mainly due to biological differences between these tumours The present method therefore seems suitable for a testing of the cytostatic effects on tumours *in vitro* However, its predictive value for cytostatic effects *in vivo* is to be further investigated

---

This work has been supported by grants from the *Swedish Cancer Society* (project No 108 B71) and *John & Augusta Persson s Foundation for Medical Scientific Research*

REFERENCES

1 *Bonting, S L & Jones M* Determination of microgram quantities of deoxyribonucleic acid and protein in tissues grown *in vitro* Arch Biochem 66 340–353, 1957

2 *Borell, U* Influence of ovarian hormones on the metabolism of nucleic acid and phospholipids in rabbit uterus Acta Endocr (Kbh) 9 141, 1952

3 *Ceriotti, G* A microchemical determination of deoxyribonucleic acid J biol Chem 198 297–303 1952

4 *Mattern, J, Volm M & Wayss, A* Untersuchungen am soliden Walker Karzinosarkom

256 der Ratte unter Einwirkung von Zytostatika Arzneimittel Forsch (Drug Res) 22 Nr 10 1721–1722, 1972
5 *Nordqvist, S* Hormonal responsiveness of human endometrial carcinoma studied *in vitro* and *in vivo* Thesis Lund Studentlitteratur, 1969
6 *Tanneberger, St* Gewebekultur und Krebschemotherapie Arch Geschwulstforsch. *31* 387–400, 1968
7 *Wolberg, W H* & *Brown, R R* Autoradiographic studies of *in vitro* incorporation of uridine and thymidine by human tissue Cancer Res 22 1113–1119, 1962

Acta path microbiol scand Section A 81, 559–568, 1973

THE EFFECT OF A PHENYLALANINE-TYROSINE LOW DIET ON THE GROWTH AND MORPHOLOGY OF TRANSPLANTABLE MALIGNANT MELANOMAS OF THE SYRIAN GOLDEN HAMSTER (*MESOCRICETUS AURATUS*)

O A Jensen, J Egeberg and J Edmund

The Eye Pathology Institute (Head S Ry Andersen),
the Anatomy Department B (Head E Landboe Christensen),
and the University Eye Department (Head E Gregersen),
University of Copenhagen, Copenhagen, Denmark

Syrian golden hamsters carrying transplantable subcutaneous melanomas were fed a diet low in phenylalanine and tyrosine a.m. Demopoulos. Tyrosine (which can be formed from phenylalanine) is the basic amino-acid in melanin production and the substrate for tyrosinase, the basic enzyme in melanin synthesis. This enzyme is considered mandatory for a large part of vital respiration of the melanoma cell Copper, an activator of tyrosinase, was reduced in the diet by chelation and precipitation. By depriving the enzyme of both its substrate (tyrosine) and its activator (copper) an effect on the metabolism and thereby on the growth of the neoplastic melanocyte was suspected No significant difference was found in the survival period of controls and diet animals when the diet was instituted after transplantation of the tumour When the diet was started weeks before transplantation, the survival period of dietarily treated animals was more than double the longest survival period of animals in the control group The long term effect of the diet on normal hamsters was the development of a grey fur Light microscopically, the tumours in the diet group showed neoplastic melanocytes with an apparently melanosome free cytoplasm, but with melanin aggregated in interstitially located melanophages the number of which was higher than in the control tumours. Ultrastructurally, melanosomes were present in the dietarily treated neoplastic melanocytes, but they were few and small The results were interpreted as an effect of the diet on the melanin metabolism

Apart from surgical and radiation therapy of malignant neoplasms, the most frequent attempts to control neoplastic growths have been by means of various antimetabolite therapies.

Little has been published regarding treatment of tumours by depletion of the cell's normal nutrients

A survey of attempts to obtain tumour re-

Received 9 xii 72 Accepted 20 ii 73

Requests for reprints should be addressed to O A Jensen, Eye Pathology Institute, Rigshospitalet Tagensvej 18, DK 2200 Copenhagen N, Denmark

gression by dietary restriction was given by *Bertino & Nixon* (1) These writers stressed that the tumour cell probably cannot resist deficiencies of particular nutrients for as long a period as normal cells, and that the neoplastic cell may have very specific requirements as to nutrients that may make it even more vulnerable to dietary or biochemically created deficiencies They indicated the melanoma cell as an example

Earlier, *Demopoulos* (5) had shown a certain degree of effect on metastatic malignant melanoma of the skin by reducing by dietary means the intake of phenylalanine and tyrosine Since we had observed promising effects from a low phenylalanine tyrosine diet in a patient with a *primary* choroidal malignant melanoma of the last eye (to be published elsewhere), the aim of the present work was experimentally to study the same diet's effect, particularly the light microscopical and ultrastructural changes, on the transplantable melanoma of the Syrian golden hamster (*Mesocricetus auratus*)

The rationale of this dietary treatment of malignant melanomas is the fact that tyrosine (into which phenylalanine can be transformed in the liver) is the basic amino-acid in melanin production, and that tyrosinase, the basic enzyme in this production, is vital to the metabolism of the melanoma cell By depriving the enzyme of its substrate and activator, an inhibiting effect on the cell metabolism should be instituted, and a concomitant effect upon the growth of the tumour might be expected

MATERIAL AND METHODS

The Syrian golden hamster (*Mesocricetus auratus*) was chosen as the experimental animal because it is an excellent bearer of a transplantable melanoma. The animals were not inbred Thirty Syrian golden hamsters bearing transplantable subcutaneous melanomas were donated by the Pathological Institute of the Medical High School at Hanover Twenty had an amelanotic melanoma and ten a melanotic malignant melanoma subcutaneously in the gluteal region This latter melanoma had undergone 9 passages, and that used in the first experiment one further passage Transplantation



Fig 1 Transplantable melanoma developed in the gluteal region of a hamster 30 days after transplantation In spite of the large size of the tumour the animals were relatively unaffected except for the last 4 5 days before death

of this melanoma was continued and at the second experiment reported below it had undergone 13 passages

Transplantation technique Under ether anaesthesia 1cc of the tumour was removed, sliced with a razor blade and homogenized in 20 ml 0 9 per cent NaCl solution Of this suspension 0 5 ml was injected subcutaneously in the gluteal region of the hamster The 'take' was around 75 per cent and a tumour developed 2 weeks to 1 month later (Fig 1)

The diet The diet consisted of a tyrosine and phenylalanine free powder 'Tyrogran', supplied by Nyegaard & Co A/S, Oslo Norway An amino-acid analysis of this is shown in Table 1 Copper as

ing water 200 mg/100 ml Copper ions are precipitated as CuS by the soluble Na_2S The penicillamine and the Tyrogran powder were made into

required This was secured by giving appropriate amounts of turnip which has a very small content of these amino-acids, (0 029 per cent ≈ 0 0011 mmol/g and 0 020 per cent 0 0018 mmol/g respectively (21)) An adequate vitamin concentrate was also given The control animals were fed a normal hamster chow This contained 2 4 mmol/g tyrosine and 3 4 mmol/g phenylalanine

Experiment I Diet after Transplantation

One hundred hamsters underwent transplantation and the diet was given for three weeks (25 animals) and two months (25 animals) respectively after transplantation Half (50 animals) were used as controls



Fig 2 Photomicrograph of 1 micron thin section of tumour tissue from a control animal Melanin granules are distributed evenly in the cytoplasm of all the tumour cells Only few melanophages are present Epon embedding Toluidine blue Lab no G 141/70 (× 800)

TABLE 1 *Amino acid Analysis of Diet Powder*

	mmol/g
Glutamine + Glutamic acid	0 65
Proline	0 40
Lysine	0 25
Leucine	0 24
Asparagine + Aspartic acid	0 19
Valine	0 19
Serine	0 18
Alanine	0 15
Glycine	0 11
Threonine	0 11
Isoleucine	0 11
Arginine	0 076
Histidine	0 055
Phenylalanine	0 0
Tyrosine	0 0

Methionine Cystein + Cystin and Tryptophan are destroyed by the method of analysis

Experiment 2 Transplantation after Diet

Fifty animals were fed the diet for two weeks. These and fifty controls were transplanted and continued their respective dietary regimens

Both experiments ran until all animals were dead All dead animals were autopsied and metastases registered

Processing of tissue Pieces of tissue from the peripheral parts of the tumours were fixed in 4 per cent phosphate buffered neutral formaldehyde and subsequently embedded in paraffin From a few moribund animals in each group tissue from peripheral parts of the tumour were removed prior to sacrificing and 1 cu millimeter cubes were immediately fixed in 6 5 per cent ice cold cacodylate buffered glutaraldehyde of pH 7 3 for 2 hours and postfixed in 2 per cent OsO4 for 2 hours, dehydrated by ethanol and embedded in epon 812 Ultrathin sections were cut with glass knives on a LKB Ultratome III, stained with lead citrate and examined in a Philips EM 300 electron microscope Routine paraffin technique was used on pieces of skin from a series of animals kept on the diet for 18 months but without transplantation of tumour tissue Further the genital organs were examined in a number of diet animals and controls.

RESULTS

Experiment 1 The total of 100 transplanted animals was reduced to 31 controls and 26 of

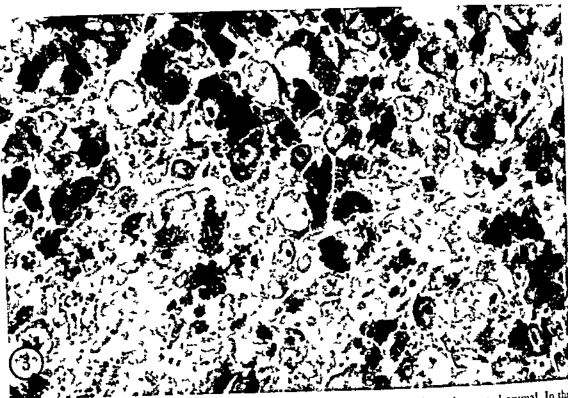


Fig 3 Photomicrograph of 1 micron thin section of tumour tissue from a dietarily treated animal In this magnification it is not possible to detect any pigment in the tumour cells, whereas the melanophages have increased in number and are heavily loaded with melanin Epon embedding Toluidine blue. Lab no. G 111/70 (× 800)

the diet group, 13 animals in each time group This reduction arose from the number of animals in which the tumour did not take and from the number that died immediately after transplantation together with the fact that only animals with significant tumours were used in the experiments No difference in survival period between the controls and the animals fed the diet was found The longest survival period in all groups was about 90 days, a third of the controls living even longer than the diet animals No difference in size of tumours number of metastases or macroscopical appearance of the tumours was found Microscopically, the tumours from the dietary group showed to a lesser degree the same changes in melanin distribution as will be described in the following experiment

Experiment 2 For the same reasons as in Experiment 1, the total of 100 transplanted animals were reduced to 29 controls and 27 animals in the dietary group which had started the dietary regimen two weeks before transplantation The number of "non takes" was the same in each group Of the 29 controls 27 died because of the tumour within 84 days and the two remaining of the group within 212 days Only 12 of the 27 from the dietary group died within 84 days, while more than half survived this period, the longest survival period being 490 days This is more than double the longest survival period in the control group The experiments were initially planned as a comparative morphological study Therefore data for statistical evaluation of the survival period in the different groups were not sufficient

Macroscopical inspection revealed no difference in size of the tumours or number of metastases Some tumours in the dietary group had an overall slight greyish colour on the cut surface, while all the controls were jet black The tumours in both groups had large central necroses It was therefore neces

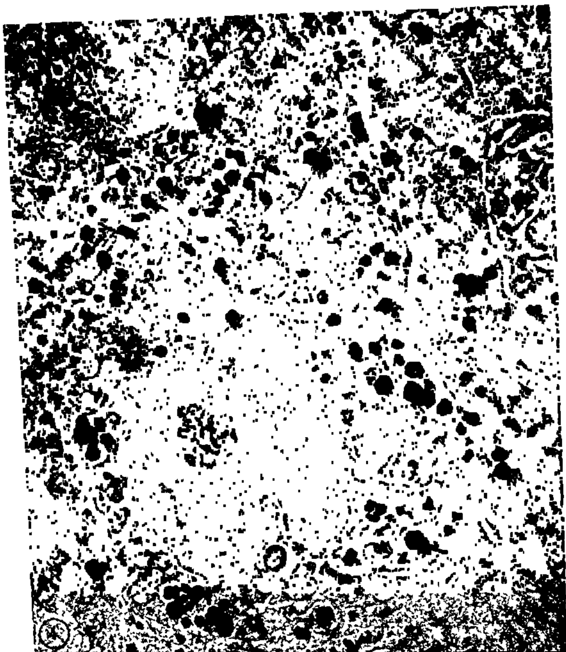


Fig 4 Electron micrograph of tumour cells from a control animal All the cells contain a large number of heavily pigmented melanosomes Lab no G 185/70 ($\times$ 14 000)

sary to examine tissue only from the solid peripheral parts

Light microscopical examination of tumour tissue revealed a difference between the two groups evident enough to separate tissue from the two groups in blind tests While the controls showed the ordinary picture of a heavily pigmented malignant melanoma with numerous small and large intracytoplasmic melanosomes and a varying, often moderate,



Fig 5 Electron mic
much less extensive
the tumour cells but they have become smaller and fewer in number Lab no

number of heavily loaded melanophages (Fig 2), the sections of tumour from the dietary group revealed that the tumour cells had a nearly melanin-free cytoplasm, while numerous heavily loaded melanophages, and more than in the control tumours, lay in bands in the intercellular connective tissue stroma giving the section a chequered pattern (Fig 3) No significant difference was found in the number of mitoses or in polymorphism



Fig 6 Normally fed hamster (A) compared with a hamster fed a tyrosine and phenylalanine low diet for 18 months (B) A visible result of the dietary treatment is that the animals developed a grey and somewhat wispy fur Note the same size and condition of the two animals.

As is the case when light microscopically amelanotic melanomas are examined by the electron microscope, the cytoplasm of the neoplastic melanocytes of the diet group was not ultrastructurally without melanosomes, but they were fewer, smaller and more scattered (Fig 4 and Fig 5) They had the usual lamellar structure and a varying melanization, as is also the case in other neoplastic melanocytes No difference in number, size or structure of mitochondria or other cytoplasmic organelles was observed

The skin from non transplanted hamsters fed the diet for a year and a half, thereby developing a grey fur (Fig 6) showed changes in the hairs and hair roots Light microscopy showed that the matrix and cortex of the hair as well as the melanocytes in the papillae were without melanin, but in contrast to normal hamster skin heavily loaded melanophages were gathered around the base of the hair bulb in the connective tissue surrounding the follicle (Fig 7)

The genital organs of animals in the dietary group (even after 18 months of dietary regimen) were of the same size and weight as in the control animals Light microscopy of testes revealed no differences, and particularly no atrophy or changes of spermiogenesis

DISCUSSSION

Malignant melanomas contain large amounts of tyrosinase, and, as is rendered probable by *Demopoulos* (2), *Demopoulos* & *Kaley* (6, 7) and *Demopoulos et al* (8, 9), a large part of vital respiration, coupled with oxidative phosphorylation, of this neoplastic cell may be dependent upon tyrosinase

On the basis of this probable dependence upon tyrosinase of the malignant melanoma cell and consequent high tyrosine requirement, *Demopoulos* reduced by dietary means the intake of phenylalanine and tyrosine, at first experimentally (3, 4) and then in five patients (5) with *metastatic* malignant melanoma of the skin A restriction of the intake of phenylalanine was necessary, since up to 70 per cent of this intake can be converted into tyrosine in the liver In three of the patients he observed a direct correlation between the institution of the diet, regression of the metastases and decrease of serum phenylalanine and tyrosine levels The dietary regimen was maintained for a maximum of five months, and the writer concluded that the dietary regimen, although nutritionally sound, was cumbersome and unpalatable Later, *Duke* & *Demopoulos* (10) and *Duke et al* (11 12) showed that tyrosinase substrate analogs with and without L- cysteine inhibited S 91 mouse melanoma in tissue culture

Ultrastructural examination of the effect of a low tyrosine and phenylalanine diet was carried out by *Mitamura et al* (20) They observed in the S-91 transplantable mouse melanoma a decreased relative turnover rate of melanosomes and swollen mitochondrial crests in addition to an increased number of fat and myelin figures They concluded that the results suggested that the mechanism of





The hairs and hair roots are black with melanin Masson Fontana

fed the diet for 18 months (compare with Fig 6) Note several melanin free hairs (arrows) and melanin aggregated in melanophages around follicles (double arrows) Masson Fontana stain Lab no 633/71 (× 150)

action of the diet was one of decreased oxidative phosphorylation, rather than a primary inhibition of protein synthesis The dietary regimen was started one week after transplantation of the tumour and maintained for six weeks The experimental and the control group each comprised three tumours.

It is worth mentioning that *Hui et al* (14 15, 16, 17, 18) observed inhibition of benign and malignant mammary tumours in C3H mice fed a phenylalanine-deficient diet, and that they considered the effect to be due to a disturbed hormonal milieu as evidenced by poor mammary gland development and presence of abnormal ovaries In a later study (19), these writers showed that the tumors

genic capabilities of doner mammary glands were not permanently impaired by a phenyl alanine deficient diet

We observed no atrophy or other morpho logical changes in the sex organs of hamsters fed the diet Males that had been fed the diet for more than a year and a half showed normal sexual activity and were mated with normal females bearing normal litters Starvation plays no role in the tumour inhibition Non transplanted hamsters fed the diet for more than a year did not lose weight The only changes found on a long term diet was the development of a grey fur (Fig 6) and corresponding changes of the hair melaniza tion (Fig 7)

In conclusion, the experiments have un doubtedly shown that the diet has an effect upon the melanogenesis in normal animals and in tumour carrying animals, the highest effect being on tumours transplanted after in stitution of the diet The aggregation of melanin in melanophages and the reduced num ber and size of melanosomes in neoplastic melanocytes do not necessarily point to a decreased melanin synthesis, but could be explained by a rapid synthesis of small melano somes with a rapid extrusion from the cyto plasm and an aggregation in melanophages In any event there is an effect on the mela nin metabolism

Although neither the fertility nor the mor phological examination of genital organs of diet treated hamsters indicated a changed hormonal balance one cannot rule out an influence via some hormonal or humoral fac tor since melanomas are considered to be conditioned tumours (13) It is also worth emphasizing that transplantable tumours and spontaneous human tumours cannot be directly compared

For the time being we are inclined to con sider the biochemical explanation given by *Demopoulos* (see above) as the most prob able

Although the present experiments have shown a certain effect on the growth of the transplantable malignant melanoma of the Syrian golden hamster and are theoretically interesting, a phenylalanine tyrosine low diet combined with an inhibition of *tyrosinase* cannot prevent the origin or development of this transplantable tumour From the present experiments it cannot be decided which of the two factors (low tyrosine or inhibited tyrosinase) were responsible for the effects observed However, it may be a beginning to the control of this fatal tumour in man by biochemical and dietary means

---

The writers are indebted to Professor, Dr *U Mohr*, Abteilung fur experimentelle Pathologie, Medizinische Hochschule, Hannover, Bundesrepublik Deutschland for kindly donating the tumour carrying hamsters

The amino acid analysis was undertaken by Biocentralen and Medicinsk Laboratorium A/S', Copenhagen

The work was supported by grants from *Komiteen til Forebyggelse af Blindhed*

REFERENCES

1 *Bertino J R & Nixon, P F* Nutritional factors in the design of more selective antitumor agents Cancer Res 29 2417–2421, 1969
2 *Demopoulos, H* Selective inhibition of S 91 mouse melanoma by phenyl lactate in vitro Bull N Y Acad Med 38 509–510, 1962
3 *Demopoulos, H B* Selective inhibition of human pigmented melanomas, in vitro and in vivo through tyrosinase inhibition Fed Proc 24 494 1965
4 *Demopoulos H B* Effects of low phenylalanine tyrosine diets on S 91 mouse melanomas J nat Cancer Inst 37 185–190 1966
5 *Demopoulos H B* Effects of reducing the phenylalanine tyrosine intake of patients with advanced malignant melanoma Cancer 19 657 664 1966
6 *Demopoulos H & Kaley, G* Inhibition of p gmented S 91 mouse melanomas, in vivo by phenyl lactate Fed Proc 21 166 1962
7 *Demopoulos H B & Kaley G* Selective inhibition of respiration of pigmented S 91 mouse melanomas by phenyl lactate and the possibly related effects on growth. J nat Cancer Inst 30 611 633 1963
8 *Demopoulos H B Gerving Martha A & Bagdoyan Helen* Selective inhibition of growth and respiration of melanomas by tyrosinase inhibitors J nat Cancer Inst 35 823–827 1965

9 *Demopoulos, H B, Kasuga, T, Channing A A & Bagdoyan, Helen* Comparison of ultra structure of B 16 and S 91 mouse melanomas, and correlation with growth patterns Lab Invest *14* 108–121, 1965

10 *Duke, P S & Demopoulos, H B In vivo* necrosis of large S 91 mouse melanomas by D2–B–Phenyllactic acid plus L–cysteine Life Sciences 6 951–957, 1967

11 *Duke, P S, Landgraf, W, Mitamura, A E & Demopoulos, H B* Study of S 91 mouse melanomas by electron paramagnetic resonance spectroscopy and tissue culture
I The effect of cysteine on blue light signals and on growth *in vitro* J nat Cancer Inst *37* 191–198, 1966

12 *Duke, P S, Yuen T G H & Demopoulos H B In vitro* growth inhibition of S 91 mouse melanomas by tyrosinase substrate analogs with an without L–cysteine Cancer Res *27* 1783–1787, 1967

13 *Furth, J* Conditioned and autonomous neoplasms A review Cancer Res *13* 477–492, 1953

14 *Hui, Y H, DeOme, K B & Briggs, G M* Inhibition of nodulogenesis in the C3H mice by a phenylalanine deficient diet Proc Amer Ass Cancer Res *11* 39, 1970

15 *Hui, Y H, DeOme, K B & Briggs, G M* Inhibition of tumorigenesis in the C3H mice by a phenylalanine-deficient diet Fed Proc *29* 428 1970

16 *Hui, Y H, DeOme, K B & Briggs G M* Inhibition of pituitary induced nodular hyperplasia in the mammary gland of C3H mice fed a phenylalanine-deficient diet J nat Cancer Inst *46* 929–939, 1971

17 *Hui, Y H, DeOme, K B & Briggs G M* Inhibition of transformation of mammary preneoplastic nodules to tumor in C3H mice fed a phenylalanine deficient diet J nat Cancer Inst *47* 245–251, 1971

18 *Hui, Y H, DeOme, K B & Briggs, G M* Inhibition of spontaneous development of hyperplastic alveolar nodules and mammary tumors in C3H mice fed phenylalanine-deficient diets J nat Cancer Inst *47* 687–695 1971

19 *Hui, Y H, DeOme, K B & Briggs, G M* The developmental, noduligenic and tumorigenic potentials of transplanted mammary glands and primary ducts from C3H mice previously fed phenylalanine-deficient diet. Cancer Res *32* 57–60, 1972

20 [illegible]
rosine restriction *in vivo* Amer J Path. *49* 309 324, 1966

21 *Orr, M L & Watt, B K* Amino acid content of foods Home economics research report No 4 Washington D C 1968, p 64

Acta path microbiol scand Section A 81, 569-576, 1973

ACTIVATION OF LYMPHOID LEUCOSIS IN CHICKS BY IRRADIATION

BENGT LAGERLOF and IVO MARES

Department of Pathology, Karolinska Sjukhuset
and State Bacteriological Laboratory, Stockholm.

Two strains of commercially bred chicks (White Leghorn and Babcock), and one strain of 'leucosis free' chicks (White Leghorn SPAFAS) were treated with whole-body irradiation This treatment enhanced the incidence of lymphoid leucosis in the commercially bred animals, with the highest dose of irradiation employed (300 rad × 4), 20 per cent of the Babcock chicks developed lymphoid leucosis after a latent period of 161-357 days, whereas none of the irradiated SPAFAS chicks which were kept in strict isolation during the whole experimental period developed leucosis One out of eight SPAFAS chicks kept in contact with 'leucosis-carrying" chicks developed lymphoid leucosis The disease was transmissible to other non irradiated chicks with cell free material The whole body irradiation is considered to have a complex effect with possible activation of latent avian tumour virus The possibility of induction of virus production in 'leucosis-free' chicks is discussed

Repeated whole body irradiation can induce thymic lymphomas in mice (e g *Kaplan* 1954, *Law* 1960) Various chemical agents are also effective in inducing thymic lymphomas or promoting the leukaemogenic effect of irradiation (*Berenblum & Trainin* 1963, *Haran-Ghera & Kaplan* 1964, *Kawamoto et al* 1958) Since leukaemogenic virus can be recovered from irradiation or chemically induced lymphomas (*Haran-Ghera* 1966 *Lieberman & Kaplan* 1959) it has been suggested that the virus remains latent in the mice until their activation is triggered by some physical or chemical stimulus (*Kaplan* 1970)

Virus of the avian leucosis group is present naturally in many strains of chickens The incidence, time of onset and clinical pattern of the diseases evoked by the virus differ in different strains The viruses are transmitted vertically and horizontally and are associated with RNA virus (*Bather* 1957)

Received 15 i 73 Accepted 21 ii 73

The aim of the present investigation was to find out whether the release of latent avian leucosis virus can be triggered by whole-body irradiation in the same way as has been shown for the mouse lymphoma system Two strains of chickens from commercial flocks known to be infected with avian leucosis virus, and one leucosis-free strain have been given fractionated whole-body irradiation at different dose levels in attempts to activate the virus The appearance of virus and viral antibodies in the plasma of the irradiated chicks has been studied by the group specific (gs) avian tumour antigen induction test and RSV focus neutralization test

EXPERIMENTAL

Fowl strains Two random bred strains of White Leghorn and Babcock chickens, purchased from commercial breeders, have been used (Vanhammar Vansbro and Vaderstad Kontrollhonseri, Vaderstad) Over 100 birds of each strain have been

kept at this laboratory for one year and a half. No lymphoid leucosis has occurred among these birds. One of the breeders reports occasional cases of lymphoid leucosis among thousands of fowls of the Babcock strain after a latent period of 7-12 months under natural conditions (Vaderstad Kontroll honseri).

No attempts have been made to isolate the two chicken strains from each other at this laboratory, and experimental chicks and control chicks have been kept in the same room under suitable experimental conditions. All experimental and control subjects were maintained on a commercial available food (Fors), and had free access to drinking water.

One commercial strain of specific pathogen free (SPAFAS Type C/O) White Leghorn chicks has also been used (Lohman, Cuxhaven, West Germany). This strain has been continously tested for the occurrence of leucosis virus by means of gs avian tumour antigen induction tests. One group of the SPAFAS strain was kept under strict isolation during the whole experimental period, whereas one group was kept in contact with the White Leghorn of the Vansbro strain and Babcock chicks during the experiments.

The age of the chicks at the start of the experiments is indicated for each separate experimental group in Table 2.

Irradiation. The chicks were irradiated with unfiltered $^{[illegible]}$Co at the doses indicated for each experimental group. Irradiation was repeated four times at one week intervals expect for one group of Babcock chicks which received one dose of 700 rad.

Intercurrent deaths. Chicks dying within two weeks post irradiation, and thus prior to the development of leucosis, have been excluded from the final calculations of the incidence of the diseases. The tables contain the "corrected numbers".

Preparation of cell free homogenate of tumour tissue. Liver or spleen tissue from leukaemic chicks was removed and frozen at –70° C immediately after death. After thawing, one gram of tissue was homogenized in 10 ml BSS in an ice bath at 30,000 rpm in a Virtis homogenizer for one minute. After centrifugation at 4 000 rpm for 10 minutes at 4° C the supernatant was inoculated into the peritoneal cavity, thymus or bursa of Fabricius in doses of 0.3 ml.

Virus strains. Rous sarcoma virus type A strain RSV (RAV 1) and type B strain RSV (RAV 2) were obtained from The Dow Chemical Co., Indianapolis, U.S.A. The strains were propagated by wing web inoculation of one day old chickens: virus stock material was prepared from the resulting tumours according to Moloney (1956) and kept frozen at –70° C. Preparations of both strains usually contained between 10^{6} to 10^{7} F.F.U. per ml.

Chick embryo cell cultures (CEC). Primary CEC cultures were prepared from 10 day-old SPAFAS embryos. The bodies of at least 10 embryos were pooled, minced and trypsinized by continuous stirring in a magnetic stirrer with 0,125 per cent trypsin for 15 min at 37° C. The released cells were centrifuged, resuspended in CEC medium and seeded in Petri dishes. After cultivation for four days at 37° C the confluent sheet of CEC was gently trypsinized and used as desired. Such cells were designated as secondary CEC. CEC medium used throughout the whole study was Eagles medium (MEM) in Earles' salt solution, supplemented with 5 per cent foetal calf serum, 5 per cent tryptose phosphate broth, and penicillin streptomycin, 50 IE and 25 IE, respectively.

Rous sarcoma virus (RSV) focus assay. Petri dishes (5 cm diameter) were seeded with 1.2 x 10 of secondary CEC and after three hours incubation at 37° in an humidified CO_2 incubator, inoculated with RSV preparation. After a further 24 hours the medium was changed for agar overlay (CEC medium with 0,9 per cent final conc of agar). On the fifth day the Petri dishes were refed with four ml CEC medium and the foci of transformed cells were counted under the microscope on the eighth day, after staining with neutral red.

Preparations of chicken plasma specimens. Attempts to isolate avian tumour virus from the blood and/or to estimate the level of antibodies by means of a neutralization test were done according to the following routine procedure. With a syringe containing 0,2 ml of a 3,8 per cent solution of sodium citrate blood was withdrawn by heart puncture from individual chicks at two or three week intervals. Plasma was withdrawn after centrifugation for 10 min at 5 000 rpm in an refrigerated centrifuge at 4° C and stored in aliquots of one ml at –70° C.

RSV focus neutralization test. The presence of neutralizing antibodies in individual plasma samples was determined in the RSV focus reduction assay with RSV (RAV 1) and RSV (RAV 2) strains. Chicken plasmas were heated to 56° C for 30 min and stored at –20° C. Individual plasmas were diluted 1:5 and 1:25 with CEC medium. Each plasma dilution was mixed with equal volume of virus preparation containing 500-1000 F.F.U. per 0.1 ml and kept at room temperature for 60 min

ml amounts (virus-dose controls in 0.1 ml amounts) into each of three Petri dishes with secondary CEC incubated at 37° C as described above (Rous sarcoma virus (RSV) focus assay). A focus count reduction greater than 50 per cent was considered as an indication for the presence of antibodies for the given virus type. If necessary, test were repeated with two fold plasma dilutions.

Virus isolation. The presence of infectious avian

TABLE 1 *Distribution of Chicks in Experimental Groups*

Group		Leucosis free	Irradiation	Corrected number	No of chicks investigated serologically	Period of observation, months
1 White Leghorn	– Vansbro	no	yes	73	0	18
Babcock	– Vaderstad	no	yes	93	14	18
2 White Leghorn	Vansbro	no	no	100	5	18
Babcock	– Vaderstad	no	no	100	5	18
3 White Leghorn in contact with group 1	SPAFAS	yes	yes	8	8	7
White Leghorn isolated	– SPAFAS	yes	yes	9	9	7

TABLE 2 *The Occurrence of Lymphoid Leucosis (LL) in Irradiated Chicks*

	Irradiation	Age at first radiation exposure (days)	Corrected no of chicks	No of LL latent period (days) in brackets
Babcock strain	100 r × 4	1	12	
	150 r × 4	1	12	1 (188)
	200 r × 4	1	30	2 (278, 357)
	300 r × 4	1	20	4 (161, 165, 301, 304)
	700 r × 1	3	19	
White Leghorn (Vansbro) strain	150 r × 4	1	11	
	200 r × 4	1	14	1 (130)
	250 r × 4	1	12	
	150 r × 4	14	12	
	200 r × 4	14	14	
	250 r × 4	14	15	1 (48)
SPAFAS	300 r × 4 in contact with gr 1	1	9	1 (290)
SPAFAS	300 r × 4 isolated	1	8	0
Babcock strain	untreated		100	0
White Leghorn (Vansbro) strain	untreated		100	0

tumour virus in chicken plasma was examined by the gs avian tumour antigen induction test in C/O type CEC cultures (*Sarma et al* 1964) Individual plasma samples were diluted 1 2, and 1 10 and inoculated in 0,4 ml amounts into three-day-old primary CEC cultures in Petri dishes The inoculated and control cultures were incubated in a CO_2 humidified incubator, trypsinized and seeded into new Petri dishes the next day, as described earlier in this section Further cell passages were performed by trypsinization of the cell sheet and seeding Petri dishes in a ratio 1 2 Confluent cell sheets from the third passage (i e after 14 days cultivation) were used for the preparation of gs antigen The cells were scraped into 0,5 ml of the remaining medium, frozen and thawed three times and kept at –70° C until further investigation The specimens were clarified by centrifugation immediately before use

The presence of gs-avian tumour antigen was detected by the complement fixation test (COFAL) (*Huebner et al* 1964) The test was performed

TABLE 3 *Attempts to Propagate LL with Cell free Material from one Babcock Chick with Postirradiation LL*

Chicken strain inoculated	Route of inoculation	No of chicks inoculated	No LL	Per Cent	Latent period (days)
White Leghorn – Vansbro	Intrapent	9	3	33	145, 161, 207
Babcock – Vaderstad	Intrapent	13	1	8	222
"	Into bursa of fabricius	7	2	29	173, 360
"	Intrathymic	7	1	14	126
Total		36	7	20	

TABLE 4 *Proportion of Serologically Positive Tests in the Various Experimental Groups*

	Infected*		Viraemia	
	number	per cent	number	per cent
Babcock, irradiated	14/14	100	6/14	43
Babcock, non irradiated	5/5	100	2/5	40
White Leghorn, non irradiated	3/5	60	3/5	60
SPAFAS, irradiated, isolated	2/9	22	0/9	0
SPAFAS, irradiated, not isolated	6/8	75	3/8	38

* "Infected denotes presence of virus and/or viral antibodies

by the technique advocated by *Sever* (1964) with 4 8 antibody units of hamster serum against the gs antigen, and 1 7 units of complement A sample was *considered to be positive if it gave 3+ or* higher specific complement fixation reaction at 1 4 or higher antigen dilution

A survey of the experimental groups is presented *in Table 1*

RESULTS

The Pathogenic Effect of Various Doses of Irradiation

Babcock strain The results of irradiation of chicks of the Babcock strain are presented in Table 2 Repeated doses of 300 rad resulted in 20 per cent incidence of lymphoid leucosis, the latent period until death ranging between 161 and 304 days Among the chicks irradiated with *200 rad × 4 two cases of* lymphoid leucosis were encountered Of 12 chicks irradiated with 150 rad × 4 one developed lymphoid leucosis whereas all 12 chicks *exposed to 100 rad × 4 were* free from leucosis

After irradiation of 19 chicks with a single dose of 700 rad no case of lymphoid leucosis appeared

Cell-free material from one of the chicks with lymphoid leucosis evoked by 200 rad × 4 was inoculated into 27 chicks of the Babcock strain *less than one* day old The total incidence of lymphoid leucosis among these chicks was 20 per cent (Table 3) One of the 13 chicks inoculated intraperitoneally developed lymphoid leucosis After inoculation into the Bursa of Fabricius, the target organ of the virus (*Cooper et al* 1968), two of the seven birds of this group de*veloped lymphoid* leucosis whereas one of seven birds inoculated intrathymically developed lymphoid leucosis

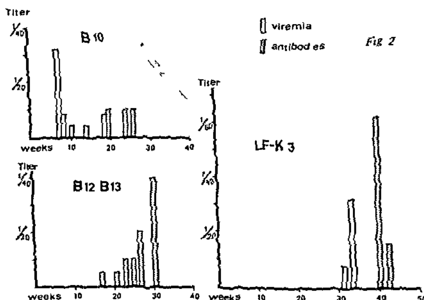
The same material inoculated intraperitoneally into a group of nine newly hatched

Fig 1

Titer
B 3
1/10
1/20
viremia
antibodies
weeks 10 20 30 40 50 60

Titer
B 6
1/20
weeks 10 20 30 40 50 60

Fig. 1 and 2 Appearance of viraemia and/or antibodies following whole body irradiation The chicks no B3 B6 B10, B12 and B13 belong to *experimental group no* 1 Chick no LFK3 belongs to experimental group no. 3, a White Leghorn SPAFAS chick kept in contact with chicks of the leucosis-carrying Babcock strain



White Leghorn chicks resulted in lymphoid leucosis in three animals, the latent period *ranging from 142 to 205 days*

White Leghorn strain (Vansbro) Only two out of a total of 78 chicks irradiated at one and 14 days of age developed lymphoid leucosis as shown in *Table 2*

SPAFAS White Leghorn strain (Lohman) Eight newly hatched chicks of the leucosis free strain exposed to 300 rad × 4 at intervals of one week were kept in strict isolation for 210 days None of these chicks developed lymphoid leucosis

Nine chicks were exposed to the same irradiation, but were kept in contact with the leucosis-carrying White Leghorn and Babcock chicks during the whole experimental period of more than one year One of these developed lymphoid leucosis 290 days after the beginning of the experiment Low levels

of viral antibodies against RSV (RAV-2) were repeatedly detected, but virus isolation experiments were negative Cell free homogenate of liver tissue from this chick was inoculated intravenously into five chicks each of the Babcock and SPAFAS White Leghorn strains None of these chicks developed lymphoid leucosis

The Appearance of Virus and Viral Antibodies in the Irradiated Chicks

The overall results are presented in Table 4

Fourteen chicks of the Babcock strain exposed to four doses of 300 rad at one-week intervals were analysed for the presence of virus and viral antibodies In all of these 14 chicks virus and/or viral antibodies were found There were great individual variations in the appearance of the virus and the antibodies, however Some representative cases are graphically demonstrated in Figs 1 and 2 Eight weeks after the first irradiation, viraemia could be demonstrated several times in chick no B6, but antibodies were never found in this bird In chick no B3 virus and antibodies could be demonstrated alternatively until 35 weeks after the irradiation when viraemia finally became persistent In chick no B10 viraemia developed seven weeks after the first irradiation This viraemia persisted for 11 weeeks when antibodies appeared Chicks no B12 and B13 perhaps exemplify a similar situation in which no virus was found in the plasma, probably due to short-termed viraemia, but a strong antibody response was present.

In the non irradiated control chicks viraemia appeared in 60 per cent and antibodies in 40 per cent of the White Leghorn strain (Vansbro) In the Babcock strain viraemia was observed in 2 of 5 animals and antibodies in 4 of 5 animals All animals in this group were infected

In two of the irradiated SPAFAS chicks, kept in isolation antibodies could be demonstrated

Seventy-five per cent of the non-isolated SPAFAS chicks were infected and viraemia was observed in 38 per cent The appearance of viraemia and antibodies in one of these chicks, LFK 3, is graphically illustrated in Fig 2

DISCUSSION

Our results indicate that irradiation of chickens causes a higher incidence of lymphoid leucosis than in birds kept under natural conditions The incidence of the disease depends on the chicken strain and the irradiation dose administered The highest over all incidence was found in the "leucosis carrying" Babcock chicks which developed lymphoid leucosis even in groups irradiated with only 150 rad × 4 The highest dose employed, namely 300 rad × 4, was most effective and resulted in 20 per cent incidence of the disease In SPAFAS chickens, however, the same dose was without any effect

Irradiation with repeated doses proved to be more effective than single dose irradiation, even at higher doses This fact fully complies with data available from experimental studies in mice (*Kaplan* 1960)

In most cases there was no appreciable reduction of the latent period of the disease compared with the few spontaneous cases developing under natural conditions Only one chicken irradiated 250 rad × 4 (White Leghorn Vansbro) developed lymphoid leucosis, the latent period being significantly reduced

In our study, the cell free material from irradiation induced lymphoid leucosis tissue induced the same disease after inoculation into non irradiated recipients This supports the hypothesis that the leukaemogenic effect of irradiation was mediated through activation of a latent virus Even the presence of antibodies and the development of lymphoid leucosis in the irradiated SPAFAS chicks which belonged to the group kept in contact with 'leucosis carrying" chicks, indicated horizontal infection during the experiment. It is a well established fact that lymphoid leucosis can be experimentally induced by different virus strains of the avian leucosis virus group such as RPL 12 and BAI A

strains (*Gross et al* 1959, *Lagerlöf* & *Sundelin* 1963)

The whole body irradiation has certainly very complex effects which involve many factors in the pathogenesis of the disease Higher doses of irradiation may be of such a magnitude that the target organ–the bursa of Fabricius has been damaged and thereafter undergone regeneration which in turn enhances its property as target organ (*Kaplan* 1960, *Chi* & *Lagerlöf* 1968) Reduced immunological capacity of the birds because of radiation damage of the lymphoid tissue is another possible factor which might influence the development of lymphoid leucosis

Virus isolation and virus neutralization tests of the ' leucosis carrying Babcock chicks showed no differences between the irradiated and the non irradiated group Signs of infection with avian leucosis virus, judged from the presence of viraemia and/or antibodies, were found in all of the chicks studied The antibody response could be due either to horizontal infection or to triggering the release of a latent virus following irradiation On the one hand, similar laboratory results, and on the other hand the different incidence of lymphoid leukosis in these two groups underline as already mentioned, the complex effect of whole body irradiation With respect to the virogene oncogene theory proposed by *Todaro* & *Huebner*, one could speculate that irradiation might have caused derepression of the oncogene which resulted in production of virus particles with a transforming capacity (*Todaro* & *Huebner* 1972)

There was a striking difference between the unirradiated Babcock and White Leghorn chicks concerning development of viraemia and antibodies This might explain some differences observed in the present study, as, for example, the higher incidence of lymphoid leucosis in the Babcock strain following irradiation or, even better results with the transmission of cell free material to the White Leghorn strain Further investigations aiming at clarifying these points are necessary

As regards this control group another interesting fact was the strong and prevalent antibody formation against RS (RAV-1) virus in the Babcock strain and the relatively late horizontal transmission of this type of virus to one chick of the White Leghorn strain Two possible explanations are available Either only type A virus was circulating in the flock, or the chicks of both strains were of the genetic type C/B, i e not suspectible to infection with the type B avian leucosis virus

As anticipated the results obtained in the group of irradiated and isolated SPAFAS chicks were significantly different

Absence of viraemia corresponded well with the fact that none of the chicks developed lymphoid leucosis However, two of these chicks developed antibodies If this were due to horizontal infection, it might be expected that a greater number of these animals would have been contaminated The chicks of the SPAFAS strain belong genetically to the C/O type and we found that approximately two thirds of these chick embryos contained gs-antigen of the avian tumour virus group (*Payne* & *Chubb* 1968) It remains a question whether the two serologically positive chicks in this experimental group might be explained by antibody response to irradiation-induced virus formation as decribed for C/O chick embryo cells cultured *in vitro* (*Weiss et al* 1971)

As regards the irradiated SPAFAS chicks which were kept in contact with the Babcock chicks, 75 per cent of the animals had either viraemia and/or antibodies The difference in this respect from the isolated group is assumed to be due mainly to contact infection from the Babcock strain The incidence of viraemia, coupled with the subsequent rise of antibody titre document the situation well and are in full accordance with the present view of horizontal transmission of the virus (*Vogt* 1965)

This work was supported by grants from the *Swedish Cancer Society* and the *Swedish Medical Research Council*

Efficient technical assistance by Miss *Yvonne Kock*, Mrs. *Britt Hall* and Mrs. *Birgitta Hagström* is gratefully acknowledged.

REFERENCES

Bather, R The nucleic acid of partially purified Rous No 1 sarcoma virus Brit J Cancer *11* 611–619, 1957

Berenblum, I & *Trainin, N* New evidence on the mechanism of radiation leukemogenesis *In* Harris, R. J C ed Cellular basis and aetiology of late somatic effects of ionizing radiation pp 41–56 London, Academic Press, 1963

Chi, C H & *Lagerlof, B* Effects of radiation damage to bone marrow on susceptibility of chicks to erythroleukemia virus Acta Radiol *7* 353–363, 1968

Cooper, M D, Payne, L N, Dent, P B, Burmester, B R & *Good, R* Pathogenesis of avian lymphoid leukosis I Histogenesis Nat. Cancer Inst *41* 373–389, 1968

Gross M A, Burmester, B R & *Walter, W G* Pathogenicity of a viral strain (RPL 12) causing avian visceral lymphomatosis and related neoplasms I Nature of the lesions J Nat Cancer Inst *22* 88–101, 1959

Haran Ghera N & *Kaplan H S* Significance of thymus and marrow injury in methan leukemogenesis. Cancer Res *24* 1926–1931, 1964

Haran Ghera, N Leukemogenic activity of centrifugates from irradiated mous thymus and bone marrow Int J Cancer *1* 81–87, 1966

Huebner, R, Armstrong D Okuyan M, Sarma P S & *Turner, H C* Specific complement fixing viral antigens in hamster and guinea pig tumors induced by the Schmidt Ruppin strain of avian sarcoma Proc Natl Acad Sci *51* 742, 1964

Kaplan, H S Experimental alteration of hos response to an occult tumor virus Induction of thymic lymphosarcomas in X irradiated mice Nat Cancer Inst Monograph no 4 141 150 1960

Kaplan H S Radiation and virus interactions in murine leukemia induction. IVth Int Congr of Rad Res. *113* 1970

Kawamoto, S, Ida, N, Kirschbaum, A & *Taylor, G* Urethane and leukemogenesis in mice. Cancer Res. *18* 725–729, 1958

Lagerlof, B & *Sundelin, P* Variations in the pathogenic effect of myeloid fowl leukaemia virus Acta Pathol Microbiol Scand *59* 129–144 1963

Law, L W Radiation carcinogenesis Advances Biol Med Phys. *7* 295–342, 1960

Lieberman, M & *Kaplan, H S* Leukemogenic activity of filtrates from radiation induced lymphoid tumours of mice Science *130* 387 388, 1959

Moloney, J B Biological studies on the Rous sarcoma virus V Preparation of improved standard lots of virus for use in quantitative investigations. J Nat Cancer Inst *16* 877, 1956

Payne, L N & *Chubb, R C* Stud es on the nature and genetic control of an antigen in normal chick embryos which reacts in the COFAL test, J Gen Virol *3* 379–391, 1968

Sarma, P S, Turner, H C & *Huebner, R. J.* An avian leukosis group specific complement fixation reaction application for the detection and assay of non cytopathogenic leukosis viruses. Virology *23* 313 321, 1964

Sever, J L Application of a microtechnique to viral serological investigations. J Immun. *88* 320–329, 1962

Todaro, G J & *Huebner R J N A S* Symposium New evidence as the bas s for increased efforts in cancer research Proc. Nat Acad Sci. USA *69* no 4, 1009 1015, April 1972

Weiss, R A, Friis, R R Katz, E & *Vogt, P K* Induction of avian tumor viruses in normal cells by physical and chemical carcinogenes Virology *46* 920–938, 1971

Vogt, P K Avian tumor viruses Adv in Virus Res *11* 361, 1965

Acta path microbiol scand Section A 81, 577 582, 1973

ALKALINE AND ACID PHOSPHATASE ACTIVITY IN SARCOID LYMPH NODES

T Palva, V Raunio and R Nousiainen

The Department of Otolaryngology, University of Oulu,
and from the State Public Health Laboratory, Oulu, Finland

Quantitative and electrophoretic analyses were performed to determine alkaline and acid phosphatase activity of nodular homogenates from 22 patients with sarcoidosis 7 patients with normal mediastinal nodes, and 4 patients with metastatic carcinomatous nodes Enzyme activity of alkaline phosphatase was stronger in sarcoidosis than in normal or carcinomatous nodes while acid phosphatase activity was similar in sarcoid and normal nodes but stronger than in carcinomatous nodes Inhibition tests showed that nodes showed acid phosphatase with a major component of erythrocyte and a minor component of prostatic type Serum alkaline phosphatase was resistant to heating at 70° C which destroyed all nodular activity Activity in the supernatants was weaker than in the homogenates The high nodular acid phosphatase activity, nearly 1,000 times stronger than in serum may contribute to an increased resistance against tubercle bacilli

In 1964 *Lurie* showed that increased resistance to tubercle bacilli infection coincided with increased levels of macrophage acid phosphatase activity In sarcoid lymph nodes we (*Palva et al* 1973) demonstrated strong acid phosphatase activity in the epithelioid and giant cell nuclei and in the cellular parts of the tubercles in general Since tuberculous aetiology of sarcoidosis is one distinct possibility (*Järvi et al* 1967, *Määttä* 1968), the sarcoid type of disease might develop from an original infection by tubercle bacilli, modified by the increased host resistance This might *in vivo* result e g in the destruction of the phospholipid fraction of the bacilli by acid phosphatase activity and, in some unknown way, lead to the hyperactive response manifest in the form of the proliferating sarcoid lymph nodes

Received 7.xii 72 Accepted 28 ii.73

Since our earlier histochemical findings of sarcoid lymph nodes were in accordance with *Lurie's* experimentally obtained results, quantitative and electrophoretic analyses of acid and alkaline phosphatase activities were undertaken, since these two enzyme activities were the strongest demonstrated in studies of various hydrolases (*Palva et al* 1973)

MATERIAL AND METHODS

Large mediastinal lymph nodes were removed from 22 patients in whom the clinical diagnosis of sarcoidosis was based on the criteria reported earlier (*Palva & Hakis* 1971) Lymph nodes from seven

three groups of 7, 8 and 7 cases, representing the acute (Group I), subacute (Group II) and chronic stages (Group III), respectively All lymph nodes

were deep frozen and kept at a temperature of −70° C until the time for analyses

For enzyme analysis the tissue specimens were thawed and homogenization was made in physiological saline with a glass homogenizer at +4° C An amount of 1 2 ml of saline was used per 1 g of fresh tissue The homogenate was centrifuged at 40,000 g for 20 min and the clear supernatant was used for quantitative phosphatase determinations and for electrophoresis Some quantitative measurements were made on a few specimens from the homogenate itself

Electrophoretic separation of iso-enzymes was carried out using a Wieme apparatus at + 12° C For alkaline phosphatase analysis, 1 per cent agar (Difco special agar) in 0 1 mol/l Veronal buffer, pH 9 5 was prepared For acid phosphatase 1 per cent agar was made in 0 05 mol/l citrate buffer, pH 6 2 Electrophoresis was performed for 20 min with a current of 30 mA per slide

The alkaline phosphatase fractions were stained using the methods of *Rawstron & Ng* (1971) β naphthyl phosphate was used as substrate in 0 1 mol/l carbonate buffer at pH 10 while Fast Blue RR was used as diazo salt The incubation period was 30 min at room temperature The fractions of acid phosphatase were stained using the method of *Smith & Whitby* (1968) 10 mg of α naphthyl phosphate and 10 mg of Fast Blue RR in 10 ml of 0 1 mol/l acetate buffer at pH 5 0 The incubation period was 2 h at room temperature

Quantitative determinations of phosphatase activity were made according to the method of *King* (1965) For acid phosphatase, the substrate phenyl phosphate was dissolved in citrate buffer pH 4 9, and for alkaline phosphatase in carbonate buffer at pH 10 1 *King's* methods were also followed in the inhibition tests for acid phosphatase using tartrate and formaldehyde except that their concentration was 10 × stronger than that used by King For alkaline phosphatase temperature inactivation tests were made by keeping the sample for 15 min either at 56° or 70° C before incubation Inhibition tests by 1 mmol L phenylalanine were done by incubation for 15 min before analysis

RESULTS

The mean values of serum alkaline and acid phosphatase activities in the different groups are given in Table 1 All averages were well inside the normal limits The relation between formaldehyde stable and tartrate labile acid phosphatase also agreed with the normal findings in all groups except subacute sarcoidosis in which there was increased tartrate lability

Table 2 shows the corresponding analyses of the supernatant after centrifugation of the homogenate at 40,000 g Alkaline phosphatase activity decreased slightly at the chronic stage of sarcoidosis, the average activity in the normal and carcinomatous lymph node homogenates was only about 1/3 of that found in acute and subacute sarcoidosis Acid phosphatase activity diminished steadily from the acute group down to the chronic In normal control nodes, the average activity cor

TABLE 1 *Mean Enzyme Activity in Serum (μmol/min × ml)*

	Sarcoidosis (stage)			Normal cases	Lung cancer cases
	I	II	III		
Alkal ne phosphatase					
Mean	0 048	0 037	0 035	0 032	0 045
SD	0 019	0 025	0 011	0 019	0 015
Acid phosphatase					
Mean	0 0036	0 0027	0 0038	0 0049	0 0049
SD	0 001	0 0016	0 0016	0 0014	0 0011
Inhibition per cent					
– tartrate	12	54	12	8	17
SD	17 9	33 8	12 7	8 7	13 4
– formaldehyde	81	97	86	85	70
SD	16 5	6 9	20 9	17 9	25 8

To obtain the levels μmol/min × 1 1 the above figures should be multiplied by 1000

TABLE 2 *Mean Enzyme Activities in Supernatants of Lymph Node Homogenates (μmol/min × g)*

	Sarco dos s (stage) I	II	III	Normal cases	Lung cancer cases
Alkal ne phosphatase					
Mean	1 9	1 9	1 3	0 7	0 5
SD	2.1	1 8	1 2	0 4	0 4
Acid phosphatase					
Mean	2 9	2 3	1 8	2 1	0 6
SD	1 6	1 6	0 8	1.5	0 5
Inhib tion per cent					
– tartrate	23	19	18	25	27
SD	8 2	9 3	13 9	3 3	4 4
– formaldehyde	81	84	76	78	83
SD	11 7	16 4	9 4	24 4	6.8

responded to that found in subacute sarcoidosis but the activity in the carcinomatous nodes was only 1/4 of the corresponding figure in active sarcoidosis The inhibition tests showed a pattern similar to serum, viz a low inhibition by tartrate and high inhibition by formaldehyde

Statistical treatment of the averages using the Student s t test showed that acid phosphatase activity was significantly higher in all groups of sarcoid lymph nodes and in the normal nodes as compared with the carcinomatous lymph node homogenates ($p \approx 0\ 02$ Average differences in alkaline phosphatase activity between sarcoid lymph node homogenates on the one hand and between the normal and carcinomatous gland homogenates on the other was just short of significant ($p > 0\ 05$)

In 11 cases belonging to the different *groups studied the enzyme activity* was measured both in the homogenate and the supernatant On an average alkaline phosphatase activity was 2 1 μmol/min × gr in the homogenate and 1 5 in the supernatant Corresponding figures for acid phosphatase were 3 0 and 1 9 mmol/min × g The percentages of inhibition by formaldehyde or tartrate were similar in both fluids

In the electrophoretic separation of serum alkaline phosphatases from the carcinoma patients only one band was found with the mobility of slow α_2-globulins (Fig 1) In the control sera and in the sera of sarcoidosis patients there was another activity zone in the β_1 area but a diffuse weaker staining covered the whole α β_1 region (Fig 2) The supernatant of sarcoid nodes and of normal lymph nodes showed similar diffuse activity in the α_2-β_1 region but a continuous though diminishing activity was present in the area of albumin (Fig 2) Two cases of carcinoma nodes had this same pattern and in two the activity ended abruptly at the α_2 zone (Fig 1)

Inhibition tests by L phenylalanine showed that the serum band disappeared totally while the glandular activity became reduced only by about 25 per cent Heating at 56° C and at 70° C did not inactivate the serum alkaline phosphatases, but the tissue activity became reduced at 56° C and disappeared after treatment at 70° C

Electrophoretic analysis of serum acid phosphatase activity (pH 6 2) showed a constant weak band moving anodically with a mobility corresponding to prealbumin at this pH (Figs 3-5) In the analyses from the supernatants the strongest band in all different groups excepting two normal nodes, was seen to migrate cathodically with a mobility of β_1-globulins (Figs 3 and 4) Another fairly broad activity band migrated anodically showing the same mobility as prealbumin In two normal lymph nodes the strongest

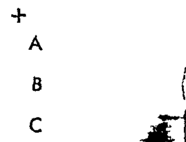


Fig 1 Electrophoretic analysis of alkaline phosphatase activity in patient serum (A) and carcinomatous lymph node homogenate (B), and in a normal lymph node homogenate (C) The sharp serum band has a mobility of slow α_2 globulins, both nodular homogenates show activity extending from α_2 to β_1 region.

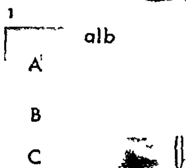


Fig 2 Electrophoretic analysis of alkaline phosphatase activity in normal human serum (A), a patient serum (B) and sarcoid node homogenate (C) In both sera there is activity in the α_2 and β_1 regions with a diffuse staining of the in between area. At the site of albumin (alb) there is non specific staining The homogenate shows a diffuse strong activity which extends to the area of albumin.

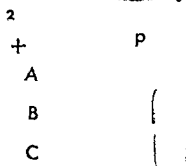


Fig 3 Electrophoretic analysis (at pH 6 2) of acid phosphatase activity in patient serum (A) and carcinoma node homogenate (B), and in a normal lymph node homogenate (C) There is one band corresponding to the mobility of pre albumin (p) in both serum and nodes while the homogenates only show a cathodic band corresponding to β_1 region This band is much stronger in the normal node than in the carcinoma node

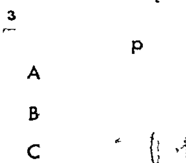


Fig 4 Electrophoretic analysis of acid phosphatase activity in normal human serum (A) and in patient serum (B) and sarcoid node homogenate (C) A weak band in the sera is seeen to correspond to pre albumin the sar
coid
band
diffuse
yond the mobility of pre albumins

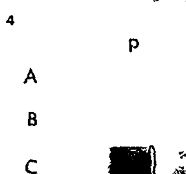


Fig 5 Electrophoretic analysis of acid phosphatase activity in normal human serum (A) and in patient serum (B) and in a normal node homogenate (C) In the homogenate the strongest activity is concentrated from the pre albumin area up to buffer front

activity was concentrated in the pre albumin area (Fig 5)

Inhibition tests by tartrate showed that this selectively inhibited the slow moving cathodic fraction which appeared only in the tissue specimens As regards formaldehyde, results were variable due to several technical reasons, but the main finding was that it inhibits mainly the faster fraction both in serum and tissue specimens, but shows only a slight effect upon the slowly moving tissue fraction

COMMENT

The present analyses show that the alkaline phosphatase activity in the sarcoid nodes was about 50 times stronger than in serum and 2 to 4 times stronger than in normal or carcinomatous lymph nodes Acid phosphatase activity was equally strong in sarcoid nodes and in normal lymph nodes, being about 1000 times stronger than in serum and four times stronger than in the carcinomatous lymph nodes The inhibition and heat inactivation tests showed that the nodular alkaline phosphatase activity had characteristics different from those found in the serum, even if both had the same electrophoretic mobility Acid phosphatase activity was mainly of the erythrocyte-type but the slowly moving fraction, appearing only in the node homogenates, was of the prostatic type This differs from the findings in two other tissues recently analysed, postauricular skin and cholesteatoma epithelium in both of which the acid phosphatase was mainly of the prostatic type (*Palva et al* 1973)

The fact that the quantitative values for the two phosphatase activities in the nodular homogenates were higher than those obtained form the supernatants is obviously due to the fact that sedimentation at 40 000 g removed part of the still lysosome bound enzymes from the supernatant

The patients own serum and the supernatant in sarcoidosis showed similar patterns on electrophoretic separation of alkaline phosphatase iso-enzymes Activity was concentrated in a fairly broad area corresponding to the mobility of α_2 β_1 region As regards acid phosphatase iso-enzymes, the nodes had a distinct band with a mobility of β_1-globulins which did not appear in sera from these patients A second band having a mobility of pre albumin appeared both in the sera and in the supernatants

We have earlier noted that the strong acid phosphatase activity of epithelioid and giant cells in the sarcoid lymph nodes accords with the findings by *Lurie* (1964) who related the increased resistance to tubercle bacilli to the high acid phosphatase activity of macrophages Studies of the viral aetiology have remained inconclusive (*Hirshaut et al* 1970, *Nikoskelainen et al* 1972) and the general morphology of tuberculous and sarcoid changes in the lymph nodes is similar These results quantify the high acid phosphatase levels in the lymph nodes, particularly during the acute stage of the disease, and there thus exists a basis for further studies of the role of acid phosphatase activity in sarcoidosis and tuberculosis

REFERENCES

Hirshaut Y Glade P, Vieira, L O B D Ainbender, E, Dvorak B & Siltzbach L E Sarcoidosis another disease associated with serologic evidence for herpes like virus infection The New Engl J Med 823 502 506, 1970

Jarvi O H Kalliomaki J L Maatta, K T Palva T K & Taiwanen A M A bacteriological and histological study of hilar lymph nodes in a series of clinical sarcoidosis La Sarcoidose Rapports de la IVe Conference Internationale Paris 12 15 Septembre 1966 Masson et Cie, 1967

King J Practical clinical enzymology Van Nostrand London 1965

Lurie M B Resistance to tuberculosis Experimental studies in native and acquired defensive mechanisms Harvard University Press Cambridge Massachusetts, 1964

Maatta K T Histological study of mediastinal lymph nodes in clinical sarcoidosis. A report of 86 cases Annales Academiae Scientiarum Fennicae Series A V Medica 138 1968

Nikoskelainen J Hannuksela M & Palva, T Fluorescent antibodies to EB (Epstein Barr) virus in sarcoidosis Scand J Clin Lab Invest 27 Suppl. 116 49 1971

Palva, T., Dammert, K. & Palva, A. On enzyme histochemistry of sarcoidosis. Acta path. microbiol. scand. Sect. A *81*: 189–194, 1973.
Palva, T. & Huhti, E. Mediastinoscopy in the evaluation of sarcoidosis. In: Mediastinoscopy. Proceedings of an International Symposium, Odense University, June 18–20, 1970. Ed. by *Otto Jepsen & H. Rahbek Sorensen*. Odense University Press 1971.
Palva, T., Raunio, V. & Nousiainen, R. Alkaline and acid phosphatase activity in postauricular skin and cholesteatoma epithelium. Acta Otolaryngol. 1973, in press.
Smith, J. K. & Whitby, L. G. The heterogeneity of prostatic acid phosphatase. Biochem. Biophys. Acta *151*: 607, 1968.
Rawstron, J. R. & Ng, S. H. Rapid electrophoresis of alkaline phosphatase isoenzymes. Clin. Chim. Acta *32*: 303, 1971.

Acta path microbiol. scand Section A 81, 583 584, 1973

BRIEF REPORTS

THE ANTI ANAEMIC PRINCIPLE IN THE LIVER FOLLOWING TOTAL GASTRECTOMY (IN PIGS) I

Svend Petri, Claus Petri and Folke Rasmussen

According to earlier studies the removal of the stomach in pigs has constantly entailed loss of the anti anaemic effect of the liver On the other hand, a recent finding has shown the presence of vitamin B_{12} in the liver to be normal after the same operation The problems concerning this discrepancy are discussed

The first collected survey on previous studies concerning the relationship between the stomach and the anti anaemic function of the liver in pigs is submitted In a study on the possible absorption of vitamin B_{12} to the blood in gastrectomized pigs, supplementary investigations of the liver were performed in two of the pigs to detect the possible presence of this vitamin The result of this latter investigation will be reported below

Previous Investigations

The relatively few experimental surgical studies of the nature concerned have been carried out by *Bence* (1933) by the team *Goodman et al* (1935) whose subsequent studies were supplemented by a number of other types of operation (1936, 1937, and in particular 1940/41), and by *Petri et al* (1941) *Maison & Ivy*s investigations do not belong here, in spite of *Ivy*s reference (1940) Experimental studies of this nature are also said to have been performed by *Bussabarger et al* (1939) and by *Bussabarger et al* (1940), but cannot be mentioned in more detail here (cf References)

The operations have consisted in total gastrectomy with end to-end anastomosis of the oesophagus to the duodenum The number of operated animals was 3 (1), 7 (5, 7), and 1 (13) respectively, the observation period 2 6 (18 36) months whereupon the pigs were sacrificed The pigs were

has not been possible to analyse the diet However, it is assumed to have been sufficient, also in iron and vitamin B respectively (1, 5, 7, 8, 14 15) Liver extract from the experimental animals has been prepared in accordance with standardized normal methods for commercial purposes (1, 5, 12, 13) 1 ml extract corresponds to 5 g liver (13)

The liver extract from the operated pigs has been tested on (usually (1)) typical, not previously treated patients with pernicious anaemia, and the i m. dosage has been 4 8 ml daily for 10 35 days (1), 2 ml daily for about 9 15 days (5) or 13 days (13) or 5 ml daily for 4-7 days (13) Subsequently in some cases long lasting administration of control preparations has shown a normal haematological response in patients with pernicious anaemia

Results

In spite of experimental differences between the named investigations, total gastrectomy in pigs has constantly entailed a gradual decrease of the amount of the anti anaemic principle in extract of the liver, leading to complete loss in about 6 8 months

Present Investigations

In order further to elucidate the relationship between the stomach and the specific liver principle, the following investigations were performed

The experimental pigs (Nos. 10 and 14) as well as the control pigs (Nos 12 and 13) were as usual of the Danish land race, about 2 months of age at gastrectomy The observation period after the operation was $18^1/_2$ months for the experimental pigs and 5½ months for the controls, and thereafter the pigs were killed. The gastrectomy was, as

Received 11 iii 73 Accepted 7 v 73

Pathological Institutes of Øresundshospitalet and

hagen, Denmark

Requests for reprints should be addressed to *Folke Rasmussen*, M D Vet Lecturer, Landbohøjskolen, 13 Bulowsvej, 1870 Copenhagen, Denmark

usual, with end to end anastomosis The diet was sufficient, but without an extra supplement of vitamin B

Determination of vitamin B_{12} in the liver was carried out terminally, as soon as possible post mortem, *direct* on the tissue A piece of hepatic tissue was homogenized to a given weight, using acetic acid/acetate buffer pH 4 5 On a sample of this tissue a vitamin B_{12} determination was performed microbiologically, using *L Leichmannii* as test organism On another sample of the homogenized tissue nitrogen determination by the Kjeldahl method was done The results are stated as vitamin B_{12} in relation to nitrogen (Determinations kindly performed by Tage Hansen, Lic pharm, Dumex Ltd, Copenhagen) The presence of vitamin B_{12} in the liver tissue was as follows

Totally gastrectomized pigs (Nos 10 and 14)
1g nitrogen ~ 17 7 μg and 12 8 μg vitamin B_{12}
Controls (Nos 12 and 13)
1 g nitrogen ~ 15 0 μg and 11 9 μg vitamin B_{12}

Result

Thus, contrary to expectations, the livers of the pigs observed for a long time after gastrectomy showed, on direct determination, a normal quantity of vitamin B_{12} equal to that found in the control pigs

Discussion

In the case of the earlier investigations traditional, postoperative causal possibilities had to be considered *Petri et al* (13) assumed that there was a relationship of dependency between the function of the fundal region and the amount of specific principle in the liver Similarly *Bence* (1) ascribed a high glycogen content in the liver extract from his gastrectomized pigs to the loss of an enzymic gastric function The loss of the liver principle following gastrectomy was demonstrated *indirectly* by the lacking haematopoietic effect of the liver extract upon patients with pernicious anaemia.

Now, extract from the livers of normal animals (pigs and oxen) is known to contain 0 5 1 5 μg/ml B_{12} Such a preparation has constantly served as an effective control on the similarly prepared–ineffective extract from the livers of the experimental animals It was concluded, therefore that the latter was devoid of vitamine B_{12}

From the experiments as a whole it is apparent that the same type of operation (total gastrectomy) has on the one hand entailed that extract from the liver must be considered devoid of vitamin B_{12}, while on the other hand direct determination has shown that the vitamin is present in normal quantities in the liver

At present the active principle in the liver is considered identical with vitamin B_{12} (6, 17, 18)

In an effort at a causal coordination of the divergent results, we are primarily going to prepare an *extract* of the last mentioned B_{12} containing livers and thereafter to perform a *direct determination* of its vitamin B_{12} content

The present studies were aided by a grant from the *P Carl Petersen Foundation* Technical aid by *Mrs Anna Jorgensen*†, *Miss Ellen Margrethe Andersen* and *Mrs Elisabeth Withpetersen* Translated from the Danish by *Mrs Anna la Cour*

References 1 *Bence, J* Z klin Med 126 127-142, 1933 —2 *Bussabarger, R A, Ivy, A C & Richter, O* "to be published', ref to by *Bussabarger, R A, Ivy, A C, Wigodsky, H C & Gunn F D* Ann int med *13* 1028–41, 1939 —3 *Bussabarger, R A, Wigodsky, H S & Ivy, A C* "in press", ref to by *Ivy, A C* 1940, "9' —4 *Geiger, A, Goodman, L & Claiborn, L N* J clin Invest *16* 666, 1937 —5 *Geiger, A J, Goodman L S & Claiborn, L N* Yale J Biol & Med *13* 259-278, 1940/41 —6 *Glass, G B J* Physiol Rev *43* 529–849, 1963 —7 *Goodman, L, Geiger, A J & Claiborn, L N* Proc soc Exper Biol & Med *32* 810–812, 1935 —8 *Goodman, L, Geiger, A & Claiborn, L N* J clin. Invest *15* 474, 1936 —9 *Ivy, A C* Am J Digst Dis *7* 500–502, 1940 —10 *Maison, G L & Ivy, A C* Proc soc exper biol & med *31* 554–556, 1933/34 —11 *Petri, S* Acta path microbiol scand Suppl *105* 1955 —12 *Petri, S, Bang, O, Kiær W & Kjerbye Nielsen, Aa* Acta med scand *116* 273–287, 1944 —13 *Petri, S, Bing, J, Nielsen E & Kjerbye Nielsen Aa* Acta med scand *109* 59–76, 1941 —14 *Petri, S, Bøggild, D, Søeborg, Ohlsen, A & Wanscher, O* Acta path microbiol scand *14* 111 120 1937 —15 *Petri, S Nørgaard, Fl & Bandier E* Acta med scand 98 117–127, 1938 —16 *Petri, S, Petri, C & Folke Rasmussen* 'To be published' —17 *Ungley C C* Proc Brit Med Assoc Cambridge June 1948 Brit Med J 2 154, 1948 —18 *West, R* Science *107* 398, 1948

Re '5' Several further papers on this subject have been listed in references as 'to be published" or "unpublished According to a communication from *Goodman* (1972), these papers never appeared because of World War II

Acta path. microbiol scand Section A. 81, 585–587, 1973

ULTRASTRUCTURE OF HUMAN MUSCLE IN MALIGNANT HYPERTHERMIA

Edith Reske Nielsen

This brief report deals with the results of an electron microscopical investigation of striated human muscle from a family (♂ 13 years old, ♀ 15 years old, ♂ 20 years old, the mother 45 years old) in which one member (♂ 13 years old) survived an anaesthetic induced malignant hyperthermia

In a previous paper (1) the clinical neurophysiological and neuropathological findings in members of this family are described and discussed The ordinary muscle biopsy from the mother was normal The two brothers and the sister revealed variations of the muscle fibre diameter, atrophic fibres, a few hyaline and basophilic segments, and a few fibres with fine vacuolization and necrosis

The electron microscopy is most surprising The ultrastructural changes are focal but present in nearly every grid and in several sections of the same grid, both in the muscle biopsy from the patient and his family, but in varying degrees.

A general view of the micrographs reveals reduced diameter of the fibres and the myofibrils Focally the structure of the muscle is severely disarranged exhibiting undulating and spiky Z-membranes and displaced A and I material (Fig A) — compared with a normal material (2)

Detailed study of the pictures displays abnormal myofibrillary pattern the Z-lines being curved and undulated the A substance increased in length and the limits of both A and I bands irregular and distorted (Fig B)

Now and then the sarcomeres reveal degenerative changes especially the I band with loss of myofilaments, and the myofibrils sometimes are widely spaced, between them and in regions from which the myofilaments have perished mitochondria, glycogen and other extremely dense granules are seen

Other pathological features can be demonstrated The Z-membrane may have a zig zag appearance referred to as 'streaming of the Z line involving one or several sarcomeres,—rods which are thought to derive from Z lines are often seen, and double Z-lines too (3, 4) Here and there the fibrils contain osmophilic bodies and lysosomes between the myofilaments or beneath the sarcoplasma membrane close to the nucleus

The muscle biopsy from the patient and his brother exhibit areas where the muscle is only made up of A material and Z membranes

The biopsy from the patient shows extremely abnormal fibres, being atrophic and the fibrils reveal areas consisting of A substance and Z membranes changing with abnormal and extremely thinned out I filaments The I substance contains abnormal Z membranes In connexion with the abnormal fibrils the organelles are pathological The mitochondria may be giant or vacuolized The sarcotubular systems are distended (Fig C and D) Empty and thickened basement membrane folds can be seen Outside the severely abnormal areas the organelles are usually normal

The micrographs of the biopsy from the brother reveal a lot of intramuscular nerves In a few axons myelin bodies are seen

The electron microscopical picture is striking and in accordance with the pattern of alterations found in muscle biopsies from another family with the same defect

The latter family consists of 141 members, 139 of which are alive Two children died from malignant hyperthermia. Twelve members, close relatives of the two deceased children reveal light microscopical and ultrastructural alterations identical with those observed in the above described family (5, to be published)

The biochemical investigation of the erythrocytes of the family from which the twelve biopsies were obtained revealed simultaneously decreased activity of the digoxine sensitive adenosine triphosphatase (S ATPase) as well as decreased osmotic resistance of the erythrocytes (6, to be published) The S ATPase is situated in cell membranes and the new observation of the low S ATPase activity is probably common to all types of cell membranes—

Received 10.iv 73 Accepted 10.iv 73

Requests for reprints should be addressed to *Edith Reske Nielsen*, Department of Neuropathology Kommunehospitalet, 8000 Aarhus C, Denmark.


fig A




Fig A Disarrangement of the sarcomeres. A A-substance, I I substance, Z Z-membrane × 6000

Fig B Abnormal constructed sarcomeres × 27000

Fig C Abnormal muscle fibre For legend see text × 6000

Fig D Details of Fig C For legend see text × 27000

---

consequently also to membranes of the muscle cell —*and the decreased myofibrillar muscle S ATPase* conclusively indicates a defect of the muscle cell membranes.

This is supported by the results obtained in Schiller's histochemical investigation of the muscle fibres (7)

On the background of these informations the interpretation of the ultrastructural findings is for the time being an open question

References 1 *La Cour, D , Juul Jensen, P & Reske Nielsen, E* Acta anaesth. Scandinav *15* 299-317, 1971 —2 *Reske Nielsen, E & Harmsen, Aa* Acta path microbiol scand Sect A, *80* 449-467, 1972 —3 *Mair, W G P & Tome, F M S* Atlas of the Ultrastructure of Diseased Human Muscle, Churchill Livingstone Edinburgh and London, 1972 —4 *Engel, A G & MacDonald, R D* In *Walton, J N , Canal, N & Scarlato, G* Muscle Diseases, Excerpta Medica, Amsterdam, *1970*, pp 71-89 —5 *Reske Nielsen, E , Kelstrup, J , Haase, J*

265-269, 1973

ORIGIN OF THE RING CHROMOSOME IN A HUMAN RECURRENT MENINGIOMA STUDIED WITH G BAND TECHNIQUE

Joachim Mark

Cytogenetically, human meningiomas are a tumour type of special interest Thus, (1) in spite of their benign character, these neoplasms mostly have an abnormal stemline, (2) numerical and/or structural deviations in group G are an almost consistent feature in abnormal stemlines, and (3) fluorescence studies have shown that pair 22 is selectively affected in group G (references in *Mark* 1973 a, b) Excepting aberrations which affect the small acrocentrics, very little is known about the details of the structural rearrangements in meningiomas This is the background for the present report which concerns the results of G band analyses of a recurrence of a meningioma with a ring chromosome The original tumour, removed 3 years earlier, was also studied chromosomally (though only with conventional staining methods, *Mark* 1971) Thus the present study also offered an opportunity to elucidate progressional characteristics of meningiomas

Materials and Methods

The recurrent, parietal meningioma was removed piecemeal from a 68 year old man The recurrence was of the syncytial type, as the original tumour and there were still no demonstrable malignant features (cf *Mark* 1971) Primary cultures were established as described by *Mark* (1973 a) The cultures were harvested 8 days later, after treatment with 0 05 μg colcemid for 4 hours The chromosome preparations were made by airdrying A week later, the slides were treated with a buffered solution of equal parts of 0 25 per cent trypsin and 0 02 per cent EDTA for 1 3 minutes and then stained with Giemsa (pH 7 3) for 12 18 minutes The chromosomes were counted in 100 cells The karyotype analyses were made by photography S was used as a symbol for stemline

Results

The chromosome counts are shown in Fig 1 The restricted spread around the prominent mode at S = 42 was mainly hypomodal. The few polyploid cells observed–all in the hypotetraploid zone–were in accord with elements derived from doub-

2 a) One variant cell with 2x = 84 had a karyotype corresponding to that of a doubled S-cell The other 7 variant cells differed from the S-cells as follows 1 cell with 2n = 43, +1D14, 2 cells with 2n = 41, –1C8, 2 further cells with 2n = 41, –1E18 1 cell with 2n = 39, –1C8, –1D15, –1F20, –1G22 +1 ring chromosome, 1 cell with 2n = 37, –1C8, 1D14, –1D15, –1F20, –1G22

The small, distal translocation on the long arm of one chromosome C9 (Fig 2 a, d, e) showed a proximal dark band and a light distal band Its origin was uncertain and suggestions seemed unwarranted in view of the chromosome losses in S

from the missing B5 (Fig 2 a c) It was also obvious that only very small distal parts of the long and short arms of the affected B5 could have been lost during the formation of the ring

One or two ring chromosomes were seen in all cells counted Except for the C9q+ and the ring-chromosomes no other marker types were observed A few cells of the modal group (not suitable for karyotype analyses) had a double sized ring but interlocked rings were not found

Discussion

The present study illustrated, unusually clearly the monoclonal origin of all elements constituting

---

Received 22 v 73 Accepted 12 vi 73

The Department of Pathology, Huddinge hospital, Huddinge and the Department of Neurosurgery, University of Lund, Lund, Sweden

Requests for reprints should be addressed to *Joachim Mark*, M D, Department of Pathology, Huddinge hospital, Huddinge, Sweden

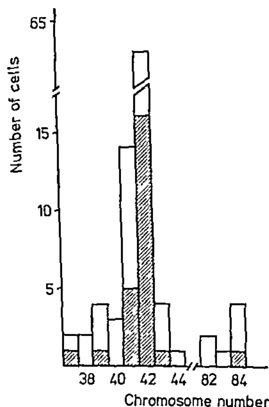


Fig 1 Chromosome counts in the recurrent meningioma Hatched areas = *karyotyped cells*

the tumour cell population This was shown by the pattern of variation among the normal chromosome types, but particularly by the consistent occurrence of the two markers, the ring chromosome and the C9q+ The pattern of deviations among normal chromosome types also elucidated important evolutionary features in meningiomas In this context, the loss of 1 G22 was the most significant finding It supported the conclusion drawn on the basis of Q band analyses, namely that there is a selective involvement of No 22 in group G in meningiomas (*Mark et al* 1972 a, b, *Zankl* & *Zang* 1972) The involvement of A1, D14 and E18 also accorded with previous observations in analyses made both with Q band technique and with conventional staining methods As regards the deviations in groups D and E, however, it remains to be clarified whether there is a preferential involvement of a certain pair The frequent involvement of C8 in the variant cells was also of interest because previous Q band analyses indicated that this pair was preferentially affected in group C and even most commonly among all pairs in all groups, except for G

The ring chromosome in the present tumour was

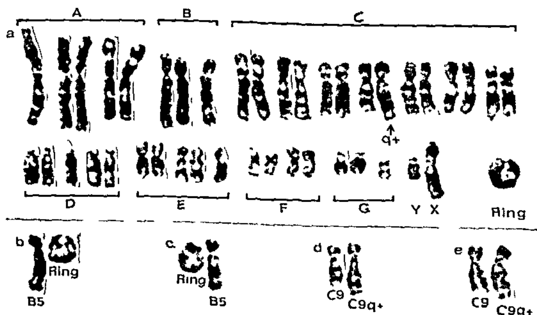


Fig 2 a Karyotype of a stemline cell, S=42, b and c One normal B5 and the ring chromosome derived from the lost B5, d and e One normal C9 and the homologue with a distal translocation, C9q+ × 2800

clearly derived from the missing B5 In the other two meningiomas reported, in which there was a ring chromosome in their S, M20 and M31 (*Mark* 1973 b), the results suggested an origin from 1A1 + 1G22 and 1D + possibly 1G, respectively Thus, there was no common denominator for the origin of the ring chromosome in the 3 cases, unless the lost G22 in the present case (together with the missing B5) had participated in the formation of the ring

The translocation, C9q+ would have passed unnoticed in preparations stained by conventional methods Though the detection of the C9q+ demonstrated the resolving capacity of the new banding techniques, it also showed their limitation, viz the remaining difficulties to trace the origin of small translocations, particularly when complex karyotypic changes have occurred

When this meningioma was studied 3 years earlier (*Mark* 1971), the tumour had the same S number as the recurrence, the S cells showed the same deviations in ordinary chromosome groups, and a ring chromosome with the same size was also present This original tumour, however, showed a greater spread around the S number and there were many hypermodal cells without ring chromosomes In the latter subgroup, the cells with only monosomy G constituted a small sideline In tissue culture this sideline took over the S in later fixations but by then it had been outgrown by normal, diploid cells

In conclusion, the in vivo progression of the present meningioma appears to be characterized by an increasing predominance of cells with ring chromosomes and an increasing frequency of cells with the S karyotype The remarkable stability of the stemline during such a long period as 3 years indicates its superior competitive capacity thus in spite of the establishment of a ring chromosome

The present study was supported by grants from the *Swedish Cancer Society*, the *University of Lund*, and *John and Augusta Persson s Foundation for Medical Research*

References 1 *Mark, J* Chromosomal aberrations and their relation to malignancy in meningiomas A meningioma with ring chromosomes. Acta path microbiol scand, A 79 193–200, 1971 —2 *Mark, J, Levan, G* & *Mitelman F* Identification by fluorescence of the G chromosome lost in human meningiomas Hereditas 71 163 168 1972a —3 *Mark, J, Mitelman, F* & *Levan G* On

1973 a, in press —5 *Mark, J* The chromosomal progression in human meningiomas. In Chromosomes and Cancer", John Wiley and sons New York, 1973 b, in press —6 *Zankl, H* & *Zang K D* Cytological and cytogenitical studies on brain tumours IV Identification of the missing G chromosome in human meningiomas as No 22 by fluorescence technique Human genetik 14 167 169 1972

Acta path microbiol scand Section A 81, 591-592, 1973

INCREASED NUCLEAR DIAMETER OF GASTRIC PARIETAL CELLS AFTER X IRRADIATION IN THE GUINEA PIG

Renato Capoferro

Increased nuclear size of intestinal epithelial cells (*Quastler & Hampton* 1962) and of gastric mucoid cells (*Helander* 1965) in irradiated mice, has been *reported* With regard to the parietal cells, no major nuclear alterations could be noted during the first 48 hours after irradiation (*Helander* 1965) Studies of parietal cell nuclei at longer time intervals after irradiation have apparently not been published

The present paper reports on a study of the diameter of parietal cell nuclei in the guinea pig diameters were measured at intervals covering from 4 to 372 days after irradiation of the stomach Fourteen irradiated and six non irradiated guinea *pigs were used Details of the technique of gastric* irradiation (1540 R at skin, field 4 5 × 5 5 cm) and of the histological procedure have been reported elsewhere (*Capoferro* 1972 a and b) No gastrin was administered during the week preceding the irradiation, since gastrin increases the DNA synthetic activity of the common progenitor cells in the

might

hour be

in all animals, including the controls Two animals *were sacrified 4 7, 14, 21, 28, 56, and 372 days* after irradiation Five longitudinal strips were cut from each stomach one from the lesser curvature, 2 from the anterior and 2 from the posterior wall Each of the strips from the anterior and posterior *wall was divided into 2 pieces thus obtaining from* every stomach 9 specimens of equal length. At least 12 parietal cell nuclei were measured in each section and the mean value of these was calculated after correction for shrinkage From these figures the average nuclear diameter for each group was calculated

The mean nuclear diameter for each group is shown in Table 1 The nuclear size of parietal cells *increased significantly after irradiation* and the highest values were found after 4 days, which is the earliest observation time in this material Furthermore, dispersion of the values was greater in the irrad ated animals than in the controls Later on a gradual decrease seemed to take place One year after irradiation no significant difference between irradiated and non irradiated animals was *found*

In general, cell response to x rays is maximal during transition from G_1 to S and at mitosis, whereas it is lowest early in G_1 and in the late part of S (*Tolmach et al* 1971) Thus, the indifferentiated multipotential progenitor cells in the neck of the glands, displaying a high mitotic activity, and, to a lesser extent, the immature superficial cells *are probably more sensitive to x ray injury* than parietal cells, which do not divide (*Ragins et al* 1968 *Willems* 1972) and probably are spared to some extent. Parietal cells, however, are reduced in number some weeks after irradiation (*Capoferro* 1972 b) This fact might be explained by either a secondary affection of the parietal cells, owing to vascular injury and oedema, or by the possibility that *the parietal cells may mirror the x ray altera*tions of the progenitor cell population, some cell cycle later Both these hypotheses might explain the apparent discrepancy between the results of the present experiment and the observation of *Helander* (1965) who noted an increased nuclear size of mucoid cells but not of parietal cells during the first two days after irradiation In fact, at longer *intervals after irradiation, new parietal cells just* derived from progenitor cells, may have a larger nuclear size This does not, however, harmonize with *the observations that long term pentagastrin* administration stimulates the production of new parietal cells (*Crean et al* 1969, *Capoferro & Nygaard* 1973), while it reduces their nuclear diameter (*Capoferro & Nygaard* 1973) Another possibility is that injured progenitor cells, which have an increased nuclear size due to x rays, generate new parietal cells with similarly increased nuclear diameter Thus, the marked increase of the parietal cell nuclear diameter observed in the present experiment 4 days after irradiation, might be an inherited sign of degeneration of the *undifferen*

Received 24 v 73 Accepted 24 v 73

The University Institute of Pathology Section A, Rikshospitalet Oslo 1, Norway

TABLE 1 *Nuclear Diameter of X-Irradiated and Non-Irradiated Parietal Cells*

	No of animals	No of sections	Nuclear diameter (μ) mean ± SEM	P[a]
Non irradiated controls	6	54	5 52 ± 0 032	–
4 days after irradiation	2	18	7 05 ± 0 18	< 0 01
7 " " "	2	18	6 52 ± 0 18	< 0 01
14 " " "	2	18	6 74 ± 0 14	< 0 01
21 " " "	2	18	6 68 ± 0 23	< 0 01
28 " " "	2	18	6 53 ± 0 17	< 0 01
56 " " "	2	18	6 26 ± 0 23	< 0 01
372 " " "	2	18	5 60 ± 0 075	NS[b]

[a] P values of irradiated animals compared with non irradiated controls.
[b] NS non significant

tiated cells in the neck area of gastric glands, caused by x rays In the mucous cells however, such changes may appear at an earlier time, corresponding to *Helander*s observations

The mechanism by which x-rays increase nuclear diameter is not clear Generally, little is known about the factors which determine nuclear size It is currently accepted that the size of the nucleus is constant for a given cell type, that immature cells have larger nuclei than mature cells, and that malignant nuclei tend to be larger and more variable in size than non malignant nuclei (*Freeman* 1964) Larger and more variable nuclear size than normal has been observed in gastric cells of patients with pernicious anaemia (*Grable et al* 1957) which is a pre malignant condition and this has been connected with maturation disorders (*Nieburgs* & *Glass* 1963) The changes in nuclear size probably do not reflect the malignant process per se, but they may mirror other biological processes Hormones seem to influence nuclear size of cells of the target organ Pentagastrin reduces the nuclear size of parietal cells in the rat, whereas antrectomy increases it (*Capoferro* & *Nygaard* 1973) Investigation of the various factors which influence the nuclear size and clarification of the mechanisms involved are needed X irradiation might represent an useful technique for the approach to this problem in animal experiments.

References 1 *Capoferro, R* Scand J Gastroent 7 599–602, 1972 a —2 *Capoferro, R* Scand J Gastroent 7 603 607, 1972 b —3 *Capoferro R* & *Nygaard, K* Scand J Gastroent In press, 1973 —4 *Crean, G P*, *Marshall, M W* & *Rumsey R D E* Gastroenterology 57 147–155, 1969 — 5 *Freeman, J A* Cellular fine structure McGraw Hill Book Co, New York 1964 —6 *Grable, E*, *Zamcheck, N*, *Jankelson, O* & *Shipp, F* Gastroenterology 32 1104–1112, 1957 —7 *Helander, H F* Rad Res 26 244–262, 1965 —8 *Nieburgs H E* & *Glass, G B J* Am J dig Dis 8 135–159 1963 —9 *Quastler, H* & *Hampton, J C* Rad Res 17 914 931, 1962 —10 *Ragins, H*, *Wincze, F*, *Liu, S M* & *Dittbrenner, M* Anat Rec. 162 99 110 1968 —11 *Tolmach L J*, *Weiss, Barbara G* & *Hopwood, L E* Fed Proc 30 1742 1751 1971 —12 *Willems, G* Digestion 6 46–63, 1972

Acta path microbiol scand Section A *81, 5*, 593–605, 1973

EOSINOPHILS IN THE BONE MARROW OF NORMAL AND CORTISOL-TREATED RATS

Quantitative and Autoradiographic Studies

FREDE BRO RASMUSSEN

Anatomy Department B (Head E Landboe-Christensen), University of Copenhagen, Denmark

Absolute counts of the eosinophil cells of the blood and bone marrow were carried out in a haemocytometer and combined with autoradiographic studies of smears of blood and bone-marrow suspensions from normal rats and cortisol treated rats A certain parallelism was found between the number of circulating eosinophil leucocytes and the eosinophils of the bone marrow Calculations showed that the bone marrow contains more than 50 times as many eosinophils as the blood Single injections of cortisol (5 mg intraperitoneally) as well as repeated injections of cortisol (5 mg every 6 hours for up to 3 days) induced blood and bone marrow eosinopenia. The decrease in the number of eosinophils in the bone marrow appears to be due primarily to a decrease of mature (non proliferating) eosinophils. During repeated injections there was continued proliferation of eosinophil cells in the bone marrow, but mitotic activity was reduced Appearances like those in the bone marrow have previously been observed in the spleen (*Bro Rasmussen* 1972 and 1973)

Most studies of the eosinophils in the blood and bone marrow have been based on differential counts and calculations from the percentage values

The present study was performed by a more direct and accurate technique in which *Yoffey* and associates' (cf int al *Yoffey* 1960) method for quantitative determination of the cell population in the bone marrow and the cellular equilibrium between the blood and bone marrow was combined with autoradiographic studies of the eosinophils in the blood and bone marrow of untreated rats and of corticoid-treated rats

Received 7 ii 73 Accepted 7 ii 73

Requests for reprints should be addressed to *Frede Bro Rasmussen* M D, Anatomy Department B University of Copenhagen, 1, Universitetsparken 2100 Copenhagen Ø, Denmark

MATERIAL AND METHODS

Animals The experimental animals as well as the controls were the same as those used for studies of eosinophils in the spleen (*Bro Rasmussen* 1972, 1973)

For the quantitative studies (routine studies) and autoradiographic studies of non steroid treated rats, 3 different groups of inbred, female albino rats were used Two groups comprised rats from Leo Pharmaceutical Products The rats in one group (A_1) weighed about 150 g (age 2 3 months) and those in the other group (A_2) 250 300 g (age 5-6 months) The 3rd group (B) comprised germ free rats weighing about 250 300 g (group B_2 of previous studies *Bro Rasmussen* 1972)

For the experiments using corticoid treatment 5-6 month-old inbred, female albino rats were used These rats, from Leo Pharmaceutical Products, weighed 250 300 g (cf group A_2) The criterion of selecting the rats was that the eosinophil count in the blood of the untreated rats had been 300 600/µl at the same hour on 3 consecutive days.

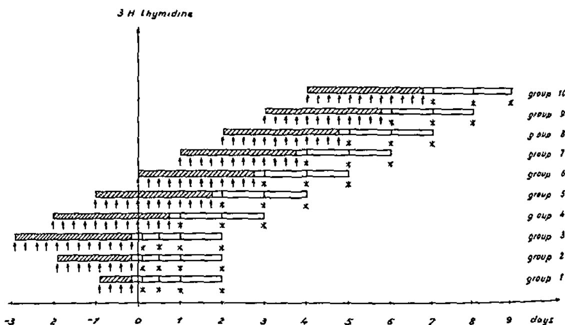


Fig 1 Experimental design (experiment 2c) The rats were divided into ten groups All rats received 0 5 μCi ^{3}H thymidine at zero time In the experimental group 5 mg cortisol was given at the times by the arrows Two rats of each group were killed at the times indicated by asterisks

For details of the treatment of the experimental rats cf the above mentioned publications

Drugs In the autoradiographic studies the rats received a single injection of radioactive thymidine in pyrogen free distilled water, specific activity 5 Ci/mM (Amersham) The rats were injected i p with 0 5 μCi per g body weight after dilution to 1 ml by 0 9 per cent saline

The corticoid treated rats received either a single injection of 5 mg cortisol (a micronized crystalline suspension in water, Leo Pharmaceutical Products) or 5 mg cortisol every 6 hours for up to 3 days (long term cortisol medication) All the injections were i p

Procedure All absolute counts of eosinophils in the peripheral blood as well as in bone marrow suspensions (*vide infra*) were done by a modification of Dunger's technique (*Andersen* 1943) Leucocyte counts and counts of the nucleated cells of the bone marrow were performed in a haemocytometer by the usual technique Smears for differential counts were stained with haematoxylin eosin

With respect to autoradiographic technique cf *Bro Rasmussen* 1972

For the investigations of the bone marrow the rats were killed with ether The femora were removed and cleaned of muscles and connective tissue The proximal and distal extremities were sawn off by a fret saw With fine scissors the shaft was split longitudinally and with an eye forceps the marrow from both parts was carefully removed and placed

not appear to offer any advantages over saline except in the determination of specific gravity (by the copper sulphate method)) As a rule the marrow could be removed as a coherent cylinder of bone marrow tissue After the bone marrow had been placed in the test tubes with saline the tubes were weighed to ascertain the weight of bone marrow in a known weight of saline

The test tubes were then placed in a mechanical shaker The marrow was easily disintegrated and formed an even cell suspension (if not the experiment was discarded) From the cell suspensions, specimens were removed for counting nucleated cells and eosinophils in haemocytometer

carry a slight error which however is prob... not of any importance partly because the specific gravity of the bone marrow is normally close to 1 (*Mechanik* 1926) and partly considering the other inaccuracies of the counting methods From the weight of the saline and bone marrow and from

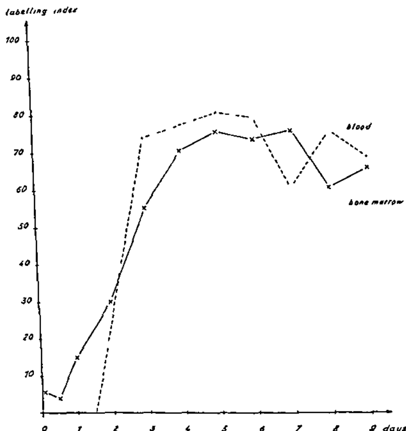


Fig 2 Labelling indices of eosinophils in the blood (investigations by Bro Rasmussen 1972) and the bone marrow in group A_1 The dots indicate the mean of 2 × 2 counts. At zero time, 0 5 μCi ^{3}H-thymidine/g body weight was given

the counts of cells, primarily the eosinophils, the number of cells per μl bone marrow was calculated

Experiments The experiments were divided into

(1) Investigations of non steroid treated rats subjected to

(a) Routine counts of eosinophils and nucleated cells in the blood and bone marrow

(b) Autoradiographic studies of eosinophils in the blood and bone marrow

(2) Corticoid-treated rats subjected to

(a) Routine studies of the number of eosinophils in the blood and bone marrow after a single injection of cortisol and after cortisol administration for up to 3 days.

(b) Autoradiographic studies of eosinophils in the blood and bone marrow after a single injection of cortisol at varying times in relation to a single injection of tritiated thymidine

(c) Autoradiographic studies of eosinophils in the blood and bone marrow after injections of cortisol for up to 3 days at varying times in relation to a single injection of tritiated thymidine

For the routine studies (experiment 1 a) of eosinophils and nucleated cells in the blood and bone marrow, 15 rats of each of the groups A_1, A_2 and B were used

The autoradiographic studies (experiment 1 b) were performed on 3 × 22 rats (groups A_1, A_2 and B) Two rats from each of the 3 groups were killed 2 hours, 12 hours, 1, 2, 3, 4, 5, 6, 7, 8, 9 days after the injection of tritiated thymidine The bone marrow was removed as described above, and at the same time the peripheral blood was studied

The experiments consisting of counts of eosinophils and nucleated cells in the blood and bone marrow in corticoid treated rats (experiment 2 a) were performed as follows Partly, a single injection of cortisol was given to 4 groups of 6 rats The rats were killed 4, 8, 16 and 24 hours after the injection Partly, 15 groups of 4 rats were in-

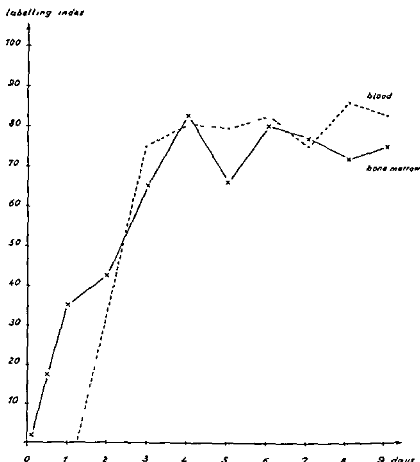


Fig 3 Labelling indices of eosinophils in the blood and the bone marrow of germ-free albino rats (The same animals as in group B_2 in the investigations by Bro-Rasmussen 1972) The dots indicate the mean of 2 × 2 counts At zero time, 0 5 μCi ^{3}H-thymidine/g body weight was given.

jected every 6 hours with 5 mg cortisol After 6 and 12 hours, 1, 1½, 2, 2½ and 3 days of cortisol injections a group of rats was killed One 8 days after the discontinuation of a 3 day cortisol medication a group of rats was killed every day (Fig 5).

The experiments consisting in autoradiographic studies after a single injection of cortisol (experiment 2 b) were performed on 6 groups of 2 rats receiving a single injection of cortisol The first group was injected with cortisol 2 hours before, the next group 8 hours after, and the subsequent groups 20, 44, 68 and 92 hours after injection of tritiated thymidine Four hours after the injection of cortisol the rats were killed In Fig 4, the times of the cortisol injections, indicated by arrows, are plotted on a curve giving the labelling indices of eosinophils in bone marrow suspensions from non-corticoid-treated rats (group A_2) The times of sacrifice are denoted by asterisks

The remaining experimental rats (experiment 2 c) were divided into 10 groups as shown in Fig 1 Three groups comprised 8 rats (groups 1-3) and 7 groups comprised 6 rats (groups 4 10) At varying times in relation to the injection of ^{3}H thymidine the cortisol medication was started 5 mg cortisol was injected every 6 hours during the period shown in the figure Two rats from groups 1-3, were killed 2, 12, 24 and 48 hours after the injection of ^{3}H thymidine, viz 6, 16, 28 and 52 hours after the cortisol medication had been discontinued As regards groups 4-10 in which all animals received cortisol medication for 66 hours, starting before or after the injection of tritiated thymidine, 2 rats were killed 6, 30 and 54 hours after the discountinuation of the cortisol medication At the time of killing, also apparent from Fig 1, blood samples were drawn and bone-marrow suspensions prepared for investigation as described above

The number of grains was counted in the autoradiographic preparations Grains were counted

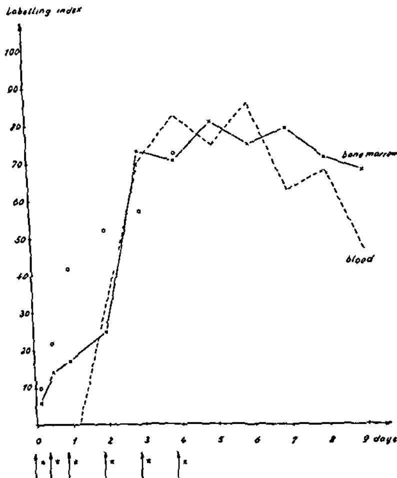


Fig 4 Labelling indices of eosinophils in the *blood* (investigations by *Bro-Rasmussen 1972*) and the bone marrow in the non steroid treated rats in group A_2 and in the bone marrow from rats in the same group 4 hours after a single injection of 5 mg cortisol
At zero time, 0 5 μCi ³H *thymidine was given. The times of injection of* cortisol are denoted by arrows below the abscissa. The asterisks denote the times at which the cortisol treated rats were killed Each group comprised 2 rats Labelling indices in the bone marrow are marked

Non steroid treated rats	×
Cortisol treated rats	o

in 25 100 cells The cells were considered to be labelled if they contained more than 3 grains.

After the single injection as well as after repeated injections of cortisol, sections of the bone marrow were prepared

RESULTS

Development was clinically normal in all the rats during the experimental period A slight fall of the haemoglobin level was observed in rats subjected to frequent blood sampling On cortisol medication lasting for more than 3 days, the rats began losing weight The *treatment was therefore restricted to 3 days*

Routine counts of circulating eosinophils in non-corticoid-treated rats (experiment 1a) in group A_1, showed during the period of acclimatization as well as during the actual experimental period, fluctuations between 50 and 150 per μl, mean 113 In group A_2 the

TABLE 1 *Eosinophil Counts in the Bone Marrow after a Single Injection of 5 mg Cortisol, 6 Rats in Each Group*

Post – inj cortisol 5 mg	0 hours (normal)	4 hours	8 hours	16 hours	24 hours
Eosinophil count pr l	78 000	48,000	53 000	87,000	69 000

findings were 300 600 pr μl, mean 401 In the germ free rats (group B), the number of eosinophils fluctuated between 10 and 120 per μl, mean 76

The leucocyte counts ranged in group A_1 from 5,000 to 13,000, in group A_2 from 9,000 to 20,000, and in group B from 3,000 to 10,000 per μl

As in the case of the spleen, the eosinophils *in the bone marrow* proved very resistant to mechanical shaking

The bone marrow contained about 2,300 000 (1,700,000–2,800,000) nucleated cells per μl without significant differences between the three groups On the other hand, the number of eosinophils differed In group A_1 there were 1 8 per cent eosinophils in relation to nucleated cells, in group A_2 3 4 per cent and in the germ free rats 1 2 per cent Absolute counts revealed in the 3 groups 41,000 78 000 and 27,500 eosinophils respectively, per μl These values were relatively higher in the bone marrow than in the spleen as far as the germ free rats were concerned (cf *Bro-Rasmussen* 1972)

The results of autoradiographic studies of the blood and bone marrow (experiment 1b) are shown in Figs 2, 3 and 4 for group A_1 B (germ-free rats) and group A respectively It is apparent from the curves that labelled eosinophils were found in the bone marrow suspensions 2, 12 and 24 hours after the injection of radioactive thymidine By way of comparison, labelling of eosinophils in the peripheral blood was not observed until 24-48 hours after the injection of thymidine Broadly speaking there was the same percentage number of labelled eosinophils in all three groups From rather more than 3 days after the injection of thymidine, the curves representing the labelling percentage in the peripheral blood and in the bone marrow are largely parallel

The eosinophil count in the bone marrow fell after a single injection of cortisol (experiment 2a) 8-16 hours after the injection, the eosinophil count had again attained the initial level (cf Table 1)

During repeated injections of cortisol for up to 3 days, the eosinophil count fell, reaching minimum levels in 2-3 days After the cortisol administration had been discontinued, the eosinophil count slowly rose, reaching normal levels in 3 4 days No definite bone marrow eosinophilia was observed after the injection (Fig 5)

When the rats were killed 4 hours after a single injection of cortisol (experiment 2b) and 2, 12 24 and 48 hours, respectively, after injection of tritiated thymidine there were labelling indices of 10 per cent, 22 per cent, 42 per cent and 52 per cent versus the corresponding findings in non corticoid treated rats of 6 per cent 14 per cent, 17 per cent and 25 per cent (Fig 4) Seventy two and 96 hours after the injection of ^{3}H thymidine there were comparable labelling indices in corticoid-treated and non corticoid treated rats (Fig 4)

Cortisol administration 5 mg/6 hours, 22 46 and 70 hours before the injection of tri...

cortisol injection of 11 per cent, 17 per cent and 9 per cent, respectively, as compared with 6 per cent in non-corticoid treated rats (Fig 6) Twelve hours after the injection of ^{3}H-thymidine (14 hours after the discontinuation of 22, 46 and 70 hours' cortisol in

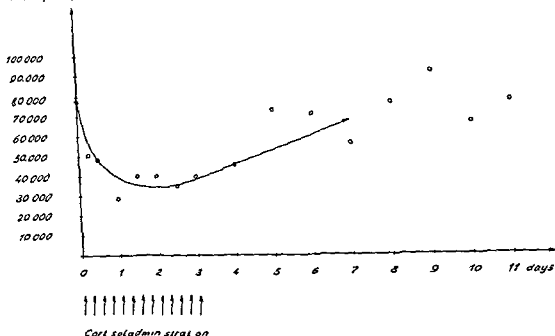


Fig 5 Counts of eosinophil cells in the bone marrow during and after cortisol administration (5 mg/6 hours) The times of injections are denoted by arows below the abscissa.

jections) there were labelling indices of 18 per cent 16 per cent and 21 per cent as compared with 14 per cent in non corticoid treated rats Twenty four and 48 hours after the injection of thymidine (26 hours and 50 hours after the discontinuation of 22, 46 and 70 hours cortisol administration) there were labelling indices of 44 per cent, 24 per cent and 28 per cent (normal, untreated rats 17 per cent), and 42 per cent, 46 per cent and 36 per cent (normal, untreated rats 25 per cent) (Fig 6)

In other words, after a single injection and during long term cortisol medication, the labelling indices were elevated as compared with those in non corticoid treated rats

When the cortisol medication (5 mg/6 hours) was started 2 days before the injection of ^{3}H thymidine and continued until 18 hours after the labelling indices 24, 48 and 72 hours after the injection of tritiated thymidine (6 30 and 54 hours after the discontinuation of the cortisol medication) proved to be 33 per cent, 35 per cent and 50 per cent (normal, untreated rats 17 per cent, 25 per cent and 73 per cent) (Fig 6) Cortisol administration, 5 mg/6 hours, from 1 day before to 42 hours after the injection of thymidine resulted in labelling indices of 50 per cent, 53 per cent and 72 per cent, respectively, when the rats were killed 48 72 and 96 hours after the injection of tritiated thymidine (normal, untreated rats 25 per cent, 73 per cent and 70 per cent) (Fig 6)

Cortisol administration for 3 days from the time of injection of ^{3}H-thymidine and later (cf experimental design Fig 1) resulted in largely the same labelling indices as in the normal material, whether the animals were sacrificed 6 hours, 30 hours, or 54 hours after the discontinuation of the cortisol medication

Attempts at counting grains per eosinophil cell in the bone marrow showed in the normal material a fall (Fig 7) in the mean grain count, largely parallel to the fall in the

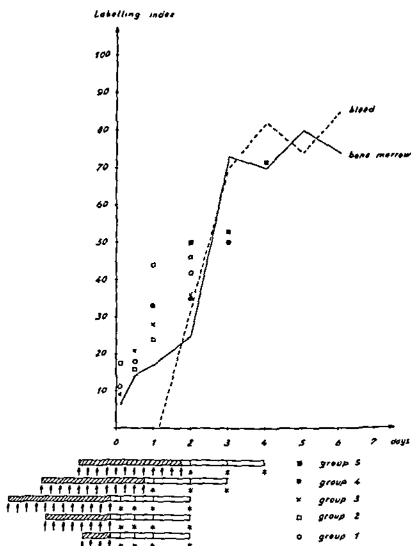


Fig 6 Diagram showing the labelling indices in the blood and the bone marrow from non steroid treated rats as in Fig 4

Labelling indices for eosinophils in the bone marrow of the rats from groups 1 5, experimental design, are plotted on the diagram fig 1 At zero time, 0 5 μCi ^{3}H thymidine/g body weight was given Cortisol injection (5 mg/6 hours) was given at the times denoted by arrows below the abscissa The asterisks denote the times at which the rats were killed

Labelling indices in the bone marrow of cortisol treated rats are marked in accordance with the symbols for each group

mean grain count per eosinophil cell in the peripheral blood (cf *Bro-Rasmussen et al* 1967) expect for the emergence time, i e the interval from the injection of thymidine to the appearance of labelled cells in the blood

After cortisol administration for 66 hours, the mean grain count was higher than that in the normal material, when investigated 6, 30 and 54 hours after the discontinuation of the cortisol administration (Fig 7)

Sections of the bone marrow showed no signs of destruction of eosinophil cells

DISCUSSION

Previous studies of the bone marrow in rats have shown a marked variation in the per-

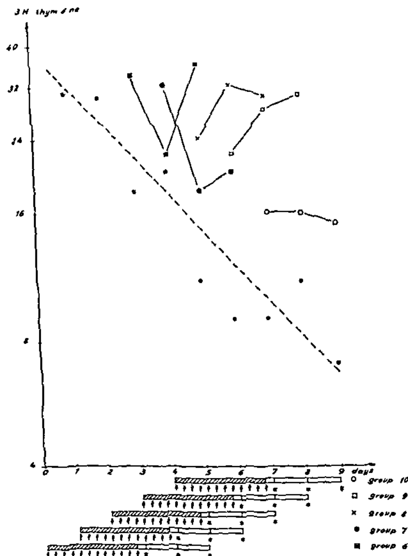


Fig 7 Average grain count per nucleus in the labelled eosinophils in the bone marrow of the normal rats (marked O) Semilogarithmic graph.
The mean grain counts for the eosinophils in the bone marrow of the rats from groups 6 10, experimental design, are plotted on the diagram fig 1 At zero time, 0 5 μCi ^{3}H thymidine/g body weight was given. Cortisol injections were given at the times denoted by arrows below the abscissa The asterisks denote the times at which the rats were killed. The mean grain count in the bone marrow of cortisol treated rats is marked in accordance with the symbols for each group

centage of eosinophil cells in relation to the nucleated cells of the bone marrow Thus, *Higgins & Marchella* (1939) found 11 75 per cent, *Töppner* (1942) 8 per cent, *Wallon & Browaeys* (1957) 7 per cent, whereas *Köhler* (1958) found only 1 98 per cent *Stasney & Higgins* (1935) found 6 98 ± 0 37 per cent (3 07-18 20) in the femur and 8 34 ± 0 66 per cent (3 04 14 25) in the ribs According to *Endicott & Ott* (1945), the activity of the bone marrow increases greatly up to the age of 1-2 months, the eosinopoietic activity ri-

sing from 4 8 ± 1 9 to 8 3 ± 5 3 The same finding was made by *Köhler* around the 36th day *Rytomaa* (1960) found an increase with age of the absolute number of eosinophil cells/100 g body weight Incidentally, his results are comparable with those obtained by Endicott & Ott

The present study showed a certain parallelism between the eosinophils in the bone marrow and the number of circulating eosinophils The count was found to increase with age, and counts were found to be higher in rats from Leo Pharmaceutical Products than in germ free rats The same relationship between the eosinophils in the blood and in the spleen has previously been found (*Bro Rasmussen* 1972) It cannot be ruled out that the cause of these findings and the previously mentioned variations in the number of eosinophils in the bone marrow in the various investigations may be that the rats have been exposed to different and varying pathogenic agents and/or allergens for varying periods of time

In the case of high peripheral eosinophil counts a parallelism between the blood and bone marrow has been found in a human series (*Bro Rasmussen* 1952)

The reported relative weight of the bone marrow in relation to body weight varies widely Thus, *Fairmann & Corner* (1934) found about 3 per cent (somewhat lower for male than female rats), *Kindred* (1942) 1 18 ± 0 53 per cent, whereas *Plum* (1943) found 3 3 per cent If the weight of the bone marrow is fixed at 2 per cent, the bone marrow of rats of group A₀ (250 300 g) contains rather more than 5 g bone marrow 85 90 per cent of which must be assumed to be active (*Kindred* (1942) 93 per cent, *End cott & Ott* (1945) 79-86 9 per cent) On this basis it may be calculated that the content of eosinophil cells in the bone marrow is in the order of 350 mill According to *Jorgensen et al* (1958), the blood volume in the rat is 5 92 ml/100 g body weight On the basis of the mean weight of the rats in group A₀, the blood volume is 16 ml, according to the stated calculations, and it may be calculated from the mean peripheral eosinophil count in the same group that there must be 6 6 mill (5-10 mill) eosinophils in the blood In other words, the bone marrow contains more than 50 times the number of eosinophils in the blood By way of comparison *Rytömaa* (1960) found 300 times as many eosinophils in the bone marrow as in the blood

I shall later return to the observed relationship between the eosinophils in the blood and the bone marrow as compared with the previously found blood transit time and the generation time of eosinophil precursors

On the present dosage of cortisol, a single injection caused a distinct reduction in the number of eosinophils in the blood as well as in the bone marrow

The peripheral fall is well known and accepted On the other hand, there is not agreement concerning the fall in number of eosinophils in the bone marrow For instance *Archer* (1963) has stated that the number of eosinophils in the bone marrow does not change during administration of ACTH and corticoids, but 12 hours after the discontinuation of such medication he observed bone-marrow eosinophilia and later blood eosinophilia *Hudson & Doeg* (1957) found an inhibited emission or production of the bone marrow eosinophils in connection with peripheral eosinopenia *Durgin & Meyer* (1951), *Quittner et al* (1951) *Esselher & Wagner* (1952), and *Uhrbrand* (1954), found no change in eosinopoiesis and an unchanged or increasing number of eosinophils in the bone marrow during the initial stages of corticoid administration After repeated injections of corticoids, *Gordon et al* (1951) *Uhrbrand* (1954), and *Wallon & Brouaeys* (1957) found bone marrow eosinopenia *Fruhman & Gordon* (1955) and *Mach et al* (1950) believe that adrenocortical hormones prevent eosinopoiesis in the bone marrow *Hudson* (1964) found an increase in the eosinophils of the bone marrow and a marked relative increase of band or lobulated forms in connection with peripheral eosinopenia

In the present study, the factor(s) inducing the peripheral eosinopenia also seem(s)

to act upon the bone marrow, resulting in bone marrow eosinopenia The same appears to apply to the spleen (*Bro Rasmussen* 1973) In a series of patients who received doses of cortocoid relatively much lower than those used in the present study, any definite fall in the number of eosinophils in the bone marrow did not occur, only a tendency towards a fall (*Bro Rasmussen* 1952)

Autoradiographic studies of non corticoid-treated rats showed labelling of eosinophils in the bone marrow during the first 24 hours at a time before labelled cells began to appear in the blood One 2 days after the injection of tritiated thymidine the first labelled cells were found in the blood, and somewhat more than 3 days after the injection of thymidine, the labelling percentage was practically the same in the blood and bone marrow The increase in the labelling indices in the bone marrow was largely parallel to the increase found previously in the spleen (*Bro Rasmussen* 1973), i e the eosinophils in the bone marrow and in the spleen follow the same pattern of proliferation During continuous infusion of tritiated thym dine *Foot* (1965) found a slower increase of the labelling indices in the spleen than in the bone marrow

Four hours after a single injection of cortisol (at a time at which bone marrow eosinopenia is manifest) the labelling index was higher than that in non corticoid treated rats from 2-48 hours after the injection of tritiated thymidine After cortisol administration (5 mg/6 hours) for 1 3 days before injection of ^{3}H *thymidine and after* 3 days cortisol administration discontinued 18 and 42 hours after the injection of thymidine the labelling indices were also higher than those in non corticoid treated rats In other words simultaneously with aneosinophilia or marked eosinopenia in the blood there occurred a fall in the absolute number of eosinophils in the bone marrow and a relative increase of labelled cells Therefore the fall in the number of eosinophils in the bone marrow *must have hit primarily the* mature and the unlabelled eosinophil cells whereas the immature eosinophils have remained relatively unaffected by the cortisol administration At all events, the proliferation continued

The finding of labelling indices of the same order in non corticoid treated rats and in rats treated for 66 hours with cortisol, discontinued 72 or more hours after the injection of ^{3}H thymidine, can indeed be interpreted only as a sign that the proliferation of eosinophil precursors has continued during the corticoid administration On the other hand, the finding *that the mean grain count per nucleus* was higher after 3 days cortisol medication than in untreated rats suggests that long term cortisol medication inhibits the mitotic activity of the eosinophils in the bone marrow The same results have been found in studies of the mitotic activity of the eosinophils in the spleen (*Bro Rasmussen* 1973)

However, the inhibition of mitotic activity *found in the present study can by no means* explain the peripheral eosinopenia during cortisol administration (cf de *Harven* 1953, *Dustin & de Harven* 1954, *Dustin* 1957, and others) A blood transit time of eosinophils of 16 24 hours, as found by *Foot* 1965, *Blenkinsopp & Blenkinsopp* 1967, and *Bro Rasmussen et al* 1967, also rules out that changes in the bone marrow–including an inhibited emission of eosinophil cells from the bone marrow–could be the main cause of the eosinopenia in the blood after a single injection of cortisol Rather it suggests a direct or indirect effect upon the eosinophils in the blood in the form of lysis or temporary removal from the circulation Eosinophils in the course of destruction were not observed in the present studies neither in the blood nor in the bone marrow

Since the number of mature eosinophils in the bone marrow and primarily the number of these cells decreases *markedly during* cortisol administration it would seem reasonable to assume that the cells of the bone marrow are affected by the same factor(s) which influence(s) the circulating eosinophils In addition long term cortisol medication appears to influence the eosinopoietic activity of the bone marrow and thereby result in a reduced release of eosinophil cells However

this last-mentioned action seems to be of less importance in explaining the peripheral eosinopenia

The mature and immature eosinophils of the spleen seem to behave in the same way as those of the bone marrow of non steroid-treated (*Bro-Rasmussen* 1972) as well as steroid-treated rats (*Bro Rasmussen* 1973)

The author wishes to acknowledge the valuable technical assistance rendered by Miss *Bianca Baumann*

REFERENCES

1 *Andersen, H C* Studies of the clinical Aspects, Etiology and Pathogenesis of Nasal Polyps and Hyperplastic Sinusitis, with Special Reference to Eosinophilia. Thesis Thaning & Appel, Copenhagen 1943

2 *Archer, R, K* The Mechanism of eosinopenia produced by ACTH and corticoids in the horse J Path Bact, *74* 387–395, 1963

3 *Blenkinsopp, E C* & *Blenkinsopp, W K* Effects of a glucocorticoid (dexamethasone) on the eosinophils in the rat J Endocrin *37*, 463–469, 1965

4 *Bro Rasmussen, F* Der ønskes en undersøgelse af ACTH's og cortisons virkninger på de hvide blodlegemer (specielt de eosinofile leucocyter) i det perifere blod i relation til knoglemarvsforandringer forårsaget af disse stoffer Aarhus Universitets Arkiv 1952

5 *Bro Rasmussen, F* Eosinopoiesis in the spleen of normal rats Scand J Haemat *9* 8–17 1972

6 *Bro Rasmussen, F* Eosinopoiesis in the spleen of normal rats following injection of cortisol Scand J Haemat In press

7 *Bro Rasmussen, F, Andersen, V* & *Henriksen, O* The kinetics of eosinophil granulocytes in rats Autoradiographic studies Scand J Haemat *4* 81 87, 1967

8 *De Harven, E* Sur l activite mitotique des myélocytes eosinophiles chez le rat colchicinisée C R. Soc Biol Paris *147* 911 913, 1953

9 *Durgin, M L* & *Meyer, R K* Effect of adreno-cortical extracts on the bone marrow eosinophils of mice Endocrinology *48* 518 524, 1951

10 *Dustin, P* Cortisone et stathomocineses, a propos de recherches sur l eosinopenie hormonale Sang 28 581–586, 1957

11 *Dustin, P* & *De Harven, E* La regulation hormonale de l eosinophilie sanguine et son mecanisme Rev Hemat *9* 307–340,1954

12 *Endicott, K M* & *Ott, M* The normal myelogram in albino rats Anat. Rec *92* 61–69 1945

13 *Esselier, A F* & *Wagner, K F* Das Verhalten der Eosinophilen in Blut und Knochenmark auf Verabreichung von adrenocorticotropem Hormon Klin Wschr *30* 705–709 1952

14 *Fairmann, E* & *Corner, G W* The bone marrow volume in the albino rat Anat Rec. 60 1–11, 1934

15 *Foot, E C* Eosinophil turnover in the rat. Brit J Haemat *11* 439–445, 1965

16 *Fruhman, G J* & *Gordon, A S* Quantitative effects of corticosterone on rat bone marrow Proc Soc Exp Biol *88* 130–131 1955

17 *Gordon, A S, Piliero, S J* & *Landau D* The relation of the adrenal to blood formation in the rat Endocrinology *49* 497–511, 1951

18 *Higgins, G M* & *Machella, T E* Bone marrow of rats made anemic by administration of sulfanilamide Anat Rec *75* 529–536 1939

19 *Hudson, G* The marrow reserve of eosinophils. Effect of cortical hormones on the foreign protein response Brit J Haemat *10*

20

Annals Med 6 228–237, 1957

21 *Jorgensen, M J, Voigt J* & *Jensen, E M* Blood volume in albino rats determined by the plasma dye method Acta physiol scand *42* 46–50, 1958

22 *Kindred, J E* A quantitative study of the hemopoietic organs of young adult albino rats Am J Anat *71* 207 243 1942

23 *Kohler, H* in *Cohrs, Jaffé* & *Meessen* Pathologie der Laboratoriumstiere Springer Verlag Berlin Gottingen & Heidelberg 1958

24 *Mach, R S, Brugger, J Della Santa R* & *Fabre, J* L hormone hypophysaire corticotrope Test de Thorn et action therapeutique dans la polyarthrite inflammatoire chronique (morbus Charcot) Schweiz med *Wschr 80* 5 11, 1950

25 *Mechanik, N* Untersuchungen uber das Gewicht des Knochenmarkes des Menschen. Zschr Ges. Anat *79* 58–99 *1926*

26 *Plum C M* Zur Granulocytopoese bei Ratten Fol Haemat. 67 119 127, *1943*

27 *Quittner, H, Wald N Sussmann L N* & *Antopol W* The effect of massive doses of cortisone on the peripheral blood and bone marrow of the mouse Blood *6* 513–521 1951

28 Rytoman T Organ Distribution and Histochemical Properties of Eosinophil Granulocytes in Rat Thesis *Acta path microbiol* scand Suppl *140* 1940

29 *Stasney, J & Higgins, G.M* A quantitative cytologic study of the bone marrow of the adult albino rat Anat Rec *63* 77–89, 1935
30 *Toppner, R* Das Myelogram der Weissen Laboratoriumratte Fol Haemat *66* 48–57, 1942
31 *Uhrbrand, H* The relation between the number of eosinophils in blood and bone marrow under ordinary conditions and under influence of ACTH Acta Haemat *11* 11–20, 1954
32 *Wallon, D & Browaeys, J* La moelle osseuse dans les eosinopénies expérimentales profondes et prolonges Sang *28* 612–619, 1957
33 *Yoffey, J M* Quantitative Cellular Haematology Thomas Springfield, 1960

Acta path microbiol scand Section A 81, 5, 606–616, 1973

ORAL GIANT CELL GRANULOMAS

A Clinical and Histological Study of 129 New Cases

LIS ANDERSEN, OLE FEJERSKOV and HANS P PHILIPSEN

Departments of Oral Pathology, The Royal Dental Colleges,
Aarhus and Copenhagen, Denmark

Ninety seven peripheral and 32 central oral giant cell granulomas have been studied clinically and histologically The distribution according to age (below and over 15 years of age) is similar in the two types of granulomas It applies to both lesions that the prevalence usually is higher in females than in males Both granulomas are most frequently found in the mandible The central giant cell granuloma occur anterior to the molar region The recurrence rate of the lesions in this material is high as compared with that observed in earlier studies The radiographic features are non specific The histopathology of the peripheral and central giant cell granulomas is fundamentally identical, both being composed of 1) a highly vascular and cellular granulation tissue containing, 2) a great number of multinuclear giant cells of foreign body type scattered in the tissue 3) extravasation by red blood cells and the presence of haemosiderin 4) occasionally bone formation, and 5) several mitoses in the stromal cells Comparative statistical analysis of the clinical findings and comparative histomorphological evaluation of the present material suggest that both types of lesions are manifestations of the same pathological condition

The giant cell granulomas have been the object of clinical, histological, and–in recent years–ultrastructural investigations (*Waldron & Shafer* 1966, *Giansanti & Waldron* 1969, *Adkins et al* 1969 a, b, *Bienengraber* 1970 *Bhaskar et al* 1971)

There are however, discrepancies between the studies which may to some extent be explained by the fact that the number of cases published are limited Thus, although the study by *Giansanti & Waldron* (1969) is based on 720 cases only 61 of these had been seen by the authors the remaining 659 being pooled from the literature This means that it is not possible to decide with certainty, whether all of these 659 cases actually were peripheral giant cell granulomas, as opinions on the histological diagnosis still are deviating

Although similar in histopathology, the pe

according to *Waldron & Shafer* (1966) the two lesions must be clearly distinguished It has become widely accepted to consider both the peripheral and central giant cell granulomas as non neoplastic or 'reparative' lesions, but there are still considerable differences in opinions on the pathogenesis of these lesions

The aim of the present study has been to present 129 new cases of peripheral and central giant cell granulomas and to report on the natural history of these lesions Because of the large number of cases presented in this study, the material was considered a su

Received 28 xi 72 Accepted 18 xii 72

Requests for reprints should be addressed to Dr *Lis Andersen*, Department of Oral Pathology, Royal Dental College, 8000 Aarhus C, Denmark

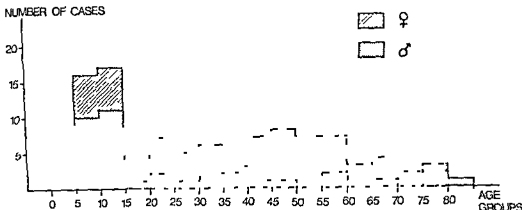


Fig 1 Peripheral giant cell granuloma Age and sex distribution of 97 cases.

table basis for a discussion of the appropriateness of maintaining a distinction between the two forms of giant cell granulomas Furthermore, the study will serve as a base line investigation for additional studies using electron microscopy

MATERIAL AND METHODS

A total of 129 tumours diagnosed as peripheral (confined to the gingival soft tissue) giant cell granulomas (97) and central (confined to the jaw bones) giant cell granulomas (32) were examined The material originated from the files of the Departments of Oral Pathology, Royal Dental Colleges, Copenhagen (89 cases) and Aarhus* (40 cases) All case histories were evaluated for pertinent data and all significant findings were tabulated If available roentgenograms were studied The histological slides were reexamined, and the diagnosis giant cell granuloma was based on the following criteria 1) a highly vascular and cellular granulation tissue containing, 2) a great number of multinuclear giant cells of foregin body type scattered in the tissue, 3) extravasation by red blood cells and the presence of haemosiderin, 4) occasionally bone formation and 5) several mitoses in the stromal cells

The tissues were fixed in a 10 per cent aqueous formalin solution or in a modified Gendre fixative (*Philipsen* 1971) Paraffin wax sections were cut 7-8 micron and stained with haematoxylin eosin van Gieson Hansen s connective tissue stain, phloxin tartrazine (*Lendrum* 1947), Gomori's aldehyde fuchsin (*Lillie* 1954) for elastic fibres, Perl s prussian blue method for iron compounds (*Romeis* 1968), periodic acid Schiff (PAS), and phosphotungstic haematoxylin (*Lillie* 1954) Tissues from ten granulomas were prefixed in a 6 25 per cent glutaraldehyde solution or in a combined paraformaldehyde glutaraldehyde solution After postfixation in 2 per cent osmium tetroxide the tissue was dehydrated and embedded in Epon or Vestopal Semithin (1 μm) sections were stained with toluidine blue and paraphenylene diamine

The results were evaluated statistically with X_2 test, always using Yates' correction for discontinuity if f = 1

* The authors are indebted to Dr *K Nielsen*, Department of Oral Pathology, Royal Dental College Aarhus for permission to include cases from his private files in this article

RESULTS

The Peripheral Giant Cell Granuloma

Age (Figure 1) The peripheral giant cell granulomas occurred in patients at ages ranging from 6 to 82 years There was a marked accumulation of cases in the group between five and 15 years

Sex Out of the 97 patients 61 were females (63 per cent) and 36 were males (37 per cent) (Figure 1) The material showed a statistical significant association between sex and age ($x^2_4 = 16{,}68$, $0{,}001 < P < 0{,}005$) Among males, the majority of patients were younger than 15 years, whereas the females showed a more even age distribution

Location In 34 cases (35 per cent), the granulomas were located to the gingiva of the maxilla, whereas the gingiva of the mandible was involved in 63 cases (65 per cent) (Fi-

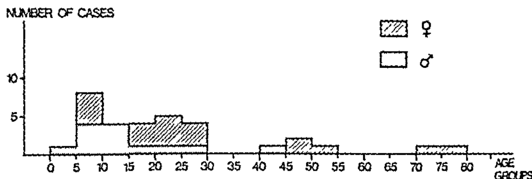


Fig 2 Central giant cell granuloma Age and sex distribution of 32 cases

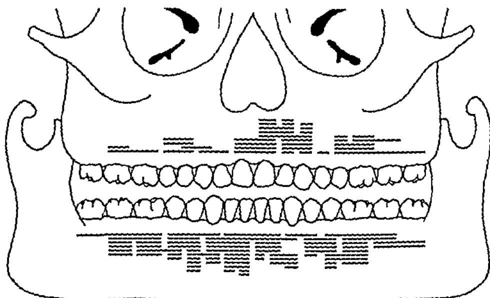


Fig 3 Peripheral giant cell granuloma Distribution and location of the 97 lesions involving the upper and lower jaws

gure 3) In 23 out of the 97 cases the granulomas developed in the alveolar mucosa in edentulous areas.

Other Clinical Aspects The size of the tumours varied from 2 mm to 40 mm in diameter, the majority being 15 mm in diameter There was no correlation between the size of the granuloma and the duration of the lesion The granuloma appeared as a well defined swelling with sessile or more rarely pedunculated basis The consistency was spongy or firm The colour of the mass varied, being either of a reddish blue or a dark red colour The surface was smooth shiny–some times somewhat humpy In 21 cases the surface was ulcerated

The lesion was generally asymptomatic but in three cases dull uncharacteristic pain was present

Tenderness to palpation was noticed in two cases, and an increased tendency to bleeding was recorded in 27 cases In some patients the presence of the granuloma resulted in local disturbances in the dental arch leading to displacement of teeth, diastemata formation delayed tooth eruption or increased tooth mobility Information about trauma to the region was available in only two cases (2 per

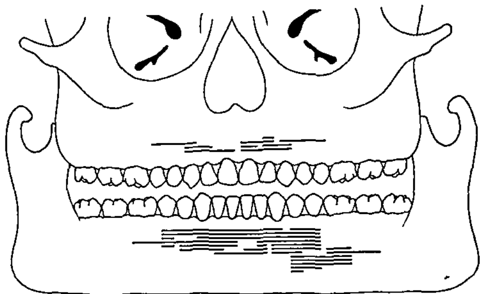


Fig 4 Central giant cell granuloma Distribution and location of the 32 lesions involving the upper and lower jaws

cent) Local irritation from dentures was present in four patients, and in another four, ill fitting dental restorations were found close to the granuloma

Duration At the time of examination the tumour had been present for periods ranging from two weeks up to three years, 70 per cent of the lesions being diagnosed within 6 months after the first sign of tumour growth had appeared

Treatment The cases were treated in local anaesthesia by excision, with or without electrocoagulation

Recurrence A check for local recurrence was made in 34 of the 97 patients Local recurrences were observed in 24 cases The recurrences were noticed at intervals of from 14 days up to five years after the primary operation

Radiographic Features Radiographs of the affected region were available in 50 cases Only in 14 cases (28 per cent) bone destruction ranging from superficial erosion (two cases) to true cavity formation (12 cases) was found Radiopaque areas were present within the soft tissue mass in 8 cases

Histological Features All 97 granulomas showed rather uniform histological features The surface was either ulcerated or covered by an ortho-or parakeratinized stratified squamous epithelium In most cases, a juxtaepithelial zone of mature collagenous connective tissue separated the epithelium from the underlying giant cell-containing granulation tissue (Figure 5) In this zone, an inflammatory cell infiltrate was often present

The giant cell granuloma consisted of a highly cellular and vascular granulation tissue containing a varying number of multinucleated giant cells of foreign body type In 12 cases (12 per cent), the granulation tissue was divided into nodules by intervening fibrous septa Extravasated erythrocytes and accumulations of haemosiderin pigment, occasionally phagocytized by macrophages, were seen in nearly all specimens (Figure 6) The haemosiderin pigment was often found at the periphery of the granuloma

The stroma cells varied in shape and stainability as well as in amount of cytoplasm (Figure 7) Spindle shaped fibroblast-like cells with only sparse cytoplasm were quite nume-

rous These cells contained lightly stained oval nuclei with distinct nucleoli or spindle-shaped dark nuclei

The giant cells showed great variations in size, morphology, and in the number of nuclei The stainability of the cytoplasm varied from light basophilia to marked eosinophilia, and variations might occur even within the same giant cell (Figure 8)

Some cells contained big ovoid and lightly stained nuclei with prominent nucleoli and sparse chromatin Often the nucleoli were dumb bell shaped Other cells contained small, darkly stained nuclei of irregular shape Cytoplasmic vacuoles of different size containing digested erythrocytes, haemosiderin pigment, and leucocytes were frequently found (Figure 9) Iron positive granules were seen both in the cytoplasm and in the vacuoles Mitotic figures in the giant cells were noticed in a few cases In many instances the giant cells showed a definite relationship to vascular channels (Figure 8)

The granulomas did not contain reticulin or elastic fibres Remnants of fibrin were encountered in the granulation tissue surrounding the giant cells (Figure 10) Bone formation with production of immature osteoid trabeculae were found in one fourth of the granulomas

The Central Giant Cell Granulomas

Age (Figure 2) The central giant cell granulomas occurred in patients at ages ranging from three to 77 years

Sex Out of the 32 patients, 19 were females (59 per cent) and 13 were males (41 per cent) The sex distribution in relation to the age of the patients is shown in Figure 2 The material showed a statistically significant correlation between sex and age, if grouped below and over 15 years of age ($x^2_1 = 5{,}56$, $0\,01 < P < 0\,025$) Among males, the majority of patients were younger than 15 years whereas most of the females were more than 15 years old

Location In 9 cases (28 per cent), the granulomas were located to the alveolar process of the maxilla, whereas the mandible was involved in 23 cases (72 per cent) The location within the jaw bones is outlined in Figure 4 In three cases, the lesions was observed in edentuluous areas of the alveolar process

Other Clinical Aspects A number of granulomas could be demonstrated only with difficulty by intraoral examination Granulomas of long duration usually appeared as bony swellings of varying consistency Crepitation due to thinning of the buccal or lingual cortical bone was noticed in a number of cases In cases where the cortical lamella were destroyed the mucous membrane covering the granulomas showed a reddish blue or reddish brown colour In two patients dull and uncharacteristic pain was noted, the remaining patients being without symptoms The presence and growth of the granuloma sometimes caused displacement or increased mobility of the teeth in the involved area Three patients showed non vital teeth in the area of the granuloma, and in one case the patient suffered from paraesthesia in the region of the mental nerve A history of trauma prior to the tumour development was recorded in one case only

Duration At the time of examination the tumours had been recognized for periods of

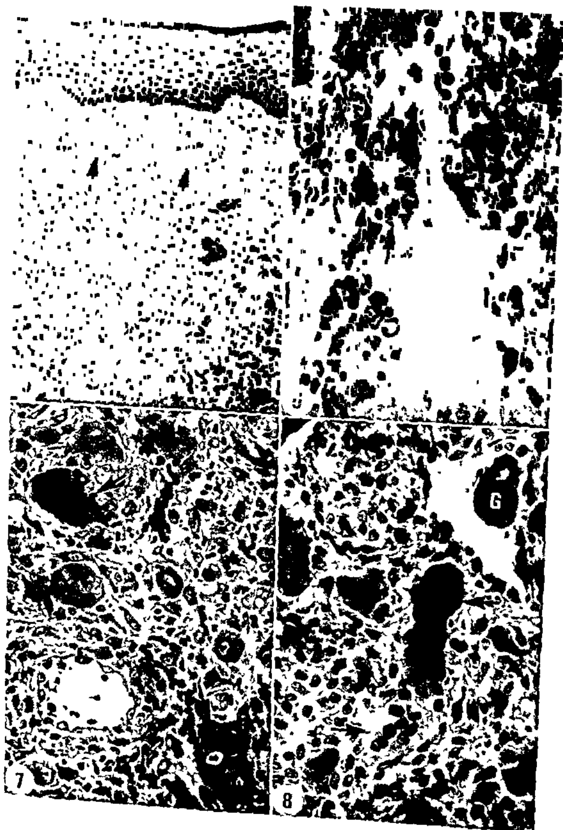
---

Fig 5 Superficial part of a peripheral giant cell granuloma The parakeratinized squamous epithelium (E) is separated from the granuloma by a zone of collagenous connective tissue (*arrows*) Multinuclear giant cells with different morphology and stainability are seen in the granuloma Haematoxylin eosin, × 110

Fig 6 Part of peripheral giant cell granuloma showing a vessel surrounded by numerous macrophages containing haemosiderin pigment (arrows) 1 μm section stained with toluidine blue, × 330

Fig 7 Part of peripheral giant cell granuloma showing close contact between giant cells and extravasated erythrocytes (arrows) 1 μm section stained with paraphenylene diamine, × 330

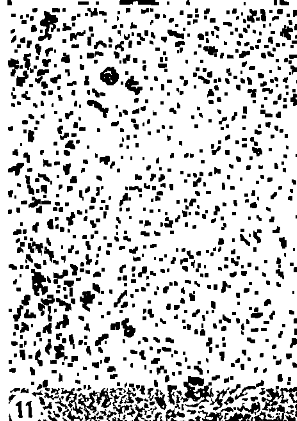
Fig 8 The stainability of the cytoplasm of the multinuclear giant cells varies within the same cell (arrows) A giant cell (G) is observed, lying free in the lumen of a capillary Haematoxylin-eosin × 560



G
7
8









three weeks up to three years, almost half of the cases being diagnosed within 6 months after the first sign of tumour growth had appeared

Treatment All cases had been treated surgically by enucleation in general or local anesthesia

Recurrence In four cases the lesion recurred locally after periods of from two to 7 months In two cases it recurred twice

Radiographic Features Radiographs were available in 26 out of the 32 cases The granuloma appeared as a radiolucent area, either unilocular (15 cases) or multilocular (11 cases) The outline of the osseous cavity was often ill defined Displacement of teeth or of toothgerms might be seen Dental root resorption was observed in two cases

Laboratory Findings Serumcalcium, phosphorous, and alkaline phosphatase values were within normal limits in all patients

Histological Features The microscopical examination of the central giant cell granulomas revealed the same histological pattern as that of the peripheral type (Figure 11) The presence of mineralized bony tissue, however, was seen more frequently in the central granulomas, being observed in about 75 per cent of the patients in this series (Figure 12)

Comparative Clinical Findings If the distribution according to sex of the peripheral and the central giant cell granulomas in this study were compared, any statistical significant difference could not be found ($x_1^2 = 0{,}021$, $0{,}80 < P < 0{,}90$) Both conditions were more frequent in females than in males Furthermore, the distribution according to age (below and over 15 years of age) of the two types of granulomas was similar ($x_1^2 = 0{,}25$, $0{,}60 < P < 0{,}70$) No significant difference in location (maxilla/mandible) of peripheral and central granulomas appeared in the present series ($x_1^2 = 0{,}25$, $0{,}60 < P < 0{,}70$) It applies to both lesions that the majority of granulomas occurred in the mandible

Fig 9 Central giant cell granuloma showing several mitotic figures in the stromal cells two of which are indicated by arrows A vacuole is seen in a multinuclear giant cell containing a phagocytized cell (arrow head) Haematoxylin eosin × 520

Fig 10 Two giant cells one of which is lying in close contact with a fibrin network (arrows) Note the marked constriction of the cytoplasm (arrow head) The nuclei of the giant cells exhibit very irregular membranes. 1 µm section stained with toluidine blue × 520

Fig 11 Part of central giant cell granuloma demonstrating a large amount of extravasated erythrocytes and numerous giant cells Phloxin tartrazine × 130

Fig 12 Osteogenesis in central giant cell granuloma. The osteoid (O) is surrounded by numerous osteoblasts. Haematoxylin-eosin × 280

DISCUSSION

The present study has shown that *the peripheral giant cell granuloma* is a lesion which may occur at any age, although one third of the patients were below 15 years of age A second peak in the fourth decade, as demonstrated by *Giansanti* & *Waldron* (1969) in their pooled material, did not appear from the present material

The granuloma is found more frequently in females than in males, which is in agreement with the results obtained by *Cooke* (1952), *Brown et al* (1956) and *Sorensen* (1958) In a series of 41 cases *Bernier* & *Cahn* (1954) found an equal sex distribution whereas *Bhaskar et al* (1959 & 1971) stated that men were affected most frequently

The lesion is more common in the mandible than in the maxilla This is in agreement with the results obtained by *Cooke* (1952) *Brown et al* (1956) and *Bhaskar et al* (1971) *Bernier* & *Cahn* (1954) and *Phillips* & *Shafer* (1955) found, however, an even distribution over the maxilla and the mandible whereas *Sorensen* (1958) noticed that the majority of lesions in this series were localized to the maxilla The minor discrepancies between the different studies as regards the age and sex distribution and location seem to be related to the number of cases examined Thus, the larger the series, the closer are the results to the findings obtained

by *Brown et al* (1956) whose studies is based on a total of 125 cases

Trauma (recent tooth extraction, decayed teeth etc) has frequently been mentioned as an important aetiological factor in the initiation of the peripheral giant cell granuloma (*Bernier* & *Cahn* (1954) and *Bhaskar et al* (1971)) In the present study, data on trauma were too scanty to allow any conclusions about the aetiological importance of this factor However, the high frequency of extractions and severely decayed teeth in the population in general makes this type of trauma less likely as a factor in view of the relative infrequency of the peripheral giant cell granuloma (about 0 5 per cent of all oral surgical accessions, according to the findings by *Giansanti* & *Waldron* (1969)) The frequency of recurrences in the present series is remarkably great as compared with that observed in other studies (*Bhaskar et al* (1971), *Sorensen* (1958) and *Austin et al* (1959)), these authors found a rate of 12 per cent, 7 7 per cent and 4 4 per cent, respectively Any explanation of this discrepancy cannot be found at present

The age and sex distribution of the *central giant cell granulomas* examined in the present study correspond closely to that stated by others (*Austin et al* (1959) *Bhaskar et al* (1959) *Kelley* & *Kay* (1965) and *Waldron* & *Shafer* (1966))

The lesion is most frequently localized to the mandible which is in agreement with observations in earlier studies (*Bhaskar et al* (1959) and *Waldron* & *Shafer* (1966)) The majority of the lesions occurs in the anterior part of the jaws, only 6 out of the 32 cases extended beyond the first permanent molar area A similar distribution has been observed by *Waldron* & *Shafer* (1966) It ought to be stressed that no granulomas involving the ramus area are not on record

The frequency of recurrence in the present series (13 per cent) is in agreement with results described in the literature

The present investigation has shown that the histopathology of the peripheral and central giant cell granuloma is fundamentally identical The only difference found is a tendency of the giant cells in a few central granulomas to accumulate into foci This is in agreement with the observations by *Waldron* & *Shafer* (1966), who found the same tendency in one third of their cases.

In the recently published WHO classification of odontogenic tumours, jaw cysts and allied lesions (*Pindborg et al* (1971)), it is stated that focal aggregations of giant cells is clearcut in some cases but in others there may be little evidence of a focal arrangement Thus, the occurrence of a focal arrangement of giant cells seems to be of little or no importance in the histopathological differential diagnosis between central and peripheral giant cell granulomas

The variation in morphology and stainability of the stromal cells corresponds to the findings by *Adkins et al* (1969b) On the basis of the stainability of the cytoplasm these authors classify the stromal cells into two types a fibroblast like type and a giant cell like type The present authors, however consider these differences in stainability merely as an expression of variations in metabolic activity rather than a representation of different cell types, as suggested by *Adkins et al* (1969b) and *Bhaskar et al* (1971) The frequent occurrence of mitotic figures among the stromal cells agrees with findings by *Giansanti* & *Waldron* (1969) and *Bhaskar et al* (1971)

The giant cells often have a very irregular outline of the cell membrane with deep invaginations In several cases the cells are segmented and nothing but a thin bridge of cytoplasm is seen to connect the segments

These findings have been interpreted as expressions of processes of cytoplasmic constriction (*Drepper et al* (1961)), but may equally well indicate a giant cell formation through fusion of cells In the granulomas mitotic figures are found in a few giant cells only These findings indicate that endomitosis hardly play any role in the production of the giant cells On the other hand, the occurrence of dumb bell shaped nuclei may indicate multinucleation by amitotic division as

proposed by *David* & *Korth* (1959) and *Drepper* & *Theman* (1961)

The pronounced phagocytic activity of the giant cells with intracellularly located iron positive pigment and leucocytes agrees well with findings in earlier studies (*Brosch* (1963), *Waldron* & *Shafer* (1966) and *Giansanti* & *Waldron* (1969)) The majority of granulomas contain pools of fibrin, frequently close to the giant cells, as shown earlier by *Kramer* (1962)

Giant cells are often found in close contact with, or lining the walls of, the vascular spaces *This intimate relation has* lead to the suggestion that the giant cells might be of endothelial or perithelial origin (*Brosch* (1957)) No investigations have so far been able to support this hypothesis

The peripheral giant cell granuloma seldom creates diagnostic problems from a histopathological viewpoint Non neoplastic gingival tissue proliferations, other than giant cell granulomas, i e calcifying fibroblastic granuloma, granuloma pyogenicum and fibrous epulis, however, may contain single giant cells (*Lee* (1968)) The small number of these cells, the lack of erythrocytes and haemosiderin pigment in these lesions usually make a distinction quite clear

A distinction between brown jaw tumours of hyperparathyroidism and central giant cell granulomas can only be made on the basis of blood chemistry The majority of aneurysmal bone cysts differ clinically from the central giant cell granulomas in that they are localized to the posterior part of the mandible especially to the ramus area Histologically, the tissue is dominated by cavernous bloodfilled spaces The histopathology of cherubism is very similar to that of the central giant cell granulomas, but may be distinguished from the latter on clinical grounds as the lesion most often occurs bilaterally in the region of the mandibular angle or, less frequently, in the posterior part of the maxilla

The literature contains conflicting statements as to a relation between the giant cell tumour of the jaws and the central giant cell granuloma *Jaffe* (1953) states that it is possible to distinguish between these lesions in the jaws if the clinical and histological findings are compared, a view which is shared by *Umiker* & *Gerry* (1954), and *Lucas* (1972)

According to the latter authors, the distinguishing histological feature of the giant cell granuloma is the relative paucity and irregular distribution of the giant cells, as compared with features of the giant cell tumour Furthermore, the size of the giant cell in the granuloma is smaller than that in the neoplasm The present study do not confirm this conception, as appears from the histological description, and the present authors find that a distinction based on histology between the giant cell tumour of the jaws and the central giant cell granuloma is difficult, if at all possible *Waldron* & *Shafer* (1966) feel convinced that central giant cell granulomas of the jaws are analogous and probably identical to benign giant cell tumours involving other bones These authors find that none of the lesions are true neoplasms, but rather reactive lesions initiated by unknown stimuli A possible relation between giant cell containing lesions of the jaws and that of other bones need further clarification

REFERENCES

1 *Adkins, K F Martinez M G & Hartley, M W* Ultrastructure of giant cell lesions A peripheral giant cell reparative granuloma Oral Surg *28* 713 723, 1969a

2 *Adkins K F, Martinez M G & Robinson, L H* Cellular morphology and relationships in giant cell lesions of the jaws Oral Surg 28 216–222 1969b

3 *Austin, L T, Dahlin, D C & Royer, R Q* Giant-cell reparative granuloma and related conditions affecting the jawbones Oral Surg 12 1285–1295, 1959

4 *Bernier J L & Cahn, L B* The peripheral giant cell reparative granuloma. J Amer dent Ass. *49* 141 148 1954

5 *Bhaskar, S N, Bernier J L & Goddy, F* Aneurysmal bone cyst and other giant cell lesions of the jaws J oral Surg *17* 30–40, 1959

6 *Bhaskar, S N, Cutright, O E, Beasley, J D & Perez, B* Giant cell reparative granuloma

(peripheral) report of 50 cases J oral Surg 29 110–115, 1971

7 *Bienengraber, V* Beitrag zur Pathogenese der Epulis Dtsch Zahn , Mund u Kieferheilk 54 211–225, 1970

8 *Brown, G N , Darlington, C G & Kupfer, S R* A clinicopathologic study of alveolar border epulis with special emphasis on benign giant cell tumor Oral Surg 9 765–775, 888–901, 1956

9 *Brosch, F* Die Riesenzellen der Epuliden abortive Kapillarsprossen oder histioretikulare Elemente Dtsch Zahn , Mund- u Kieferheilk 27 15–28, 1957

10 *Brosch, F* Zur Frage einer resorptiven Funktion der Riesenzellen der Riesenzellenepuliden Deutsch Zahnarztl Z 5 236–242, 1963

11 *Cooke, B E D* Giant-cell epulis, histogenesis and natural history Brit dent J 93 13–16, 1952

12 *David, H & Korth, I* Submikroskopische Untersuchungen zur Struktur und formalen Genese der Riesenzellen der Epulis gigantocellulares Frankfurt Z Path 70 89–97, 1959

13 *Drepper, H & Themann, H* Elektronenmikroskopische und histochemische Untersuchungen menschlicher Riesenzellepuliden Frankfurt Z Path 71 203–220, 1961

14 *Drepper, H , Themann, H & Wannenmacher, E* Untersuchungen der sublichtmikroskopischen Struktur der Riesenzellepulis Dtsch Zahn , Mund u Kieferheilk 35 1 18 1961

15 *Giansanti, J S & Waldron, C A* Peripheral giant cell granuloma review of 720 cases J oral Surg 27 787 791, 1969

16 *Jaffe, H L* Giant cell reparative granuloma, traumatic bone cyst and fibrous (fibro-osseous) dysplasia of the jaws Oral Surg 6 159–175 1953

17 *Killey H C & Kay, L W* Giant cell lesions of the jaws J int Coll Surg 44 262–272 1965

18 *Kramer, I R H* Oral pathology in the child Int Acad oral Path , New York 1962 p 48–58

19 *Lee, K W* The fibrous epulis and related lesions Granuloma pyogenicum, pregnancy tumour' , fibro-epithelial polyp and calcifying fibroblastic granuloma A clinicopathological study Periodontics 6 277–292 1968

20 *Lillie, R D* Histopathologic Technic and Practical Histochemistry The Blakiston Division, McGraw Hill Book Co , New York 1954 pp 344–345, 364–365

21 *Lendrum, A C* The phloxin tartrazine method as a general histological stain and for the demonstration of inclusion bodies J Path. Bact 59 399–404, 1947

22 *Lucas, R B* Pathology of Tumours of the Oral Tissues, 2 ed Churchill Livingstone Edinbourgh and London 1972 p 242–246

23 *Philipsen, H P* The palatal mucosa in guinea pigs Thesis, Royal Dental College, Aarhus 1971 pp 27, 43–44, 93

24 *Phillips, R L & Shafer, W G* An evaluation of the peripheral giant cell tumor J Period 26 216–222, 1955

25 *Pindborg, J J , Kramer, I R H & Torloni H* Histological typing of odontogenic tumours, jaw cysts and allied lesions WHO International histological classification of tumours. Nr 5, Geneva 1971 p 38

26 *Romeis, B* Mikroskopische Technik 16 ed R Oldenbourg Verlag, Munchen, Wien 1968 p 312

27 *Sørensen, H* Giant cell epulis. J Laryng 72 546–557, 1958

28 *Umiker, W & Gerry, R G* Pseudo giant cell tumor (reparative granuloma) of the jaw Oral Surg 7 113–123, 1954

29 *Waldron, C A & Shafer, W G* The central giant cell reparative granuloma of the jaws. An analysis of 38 cases Amer J clin. Path. 65 437–447, 1966

Acta path microbiol scand Section A 81, 5, 617–629, 1973

ORAL GIANT CELL GRANULOMAS

An Enzyme Histochemical and Ultrastructural Study

L Andersen, T Arwill, O Fejerskov, G Heyden and H P Philipsen

Departments of Oral Histopathology, Royal Dental College, Aarhus, Denmark and Faculty of Odontology, University of Goteborg, Sweden and Department of Electron Microscopy, Royal Dental College, Aarhus, Denmark

Oral giant cell granulomas (8 peripheral and 4 central) have been studied histochemically for $NADH_2$-diaphorase, $NADPH_2$ diaphorase, glucose-6 phosphate dehydrogenase, glutamate dehydrogenase, adenosine triphosphatase, leucine aminopeptidase, and acid phosphatase activity No differences in cellular enzyme activity are found between the peripheral and the central variety The giant cells are positive for all the enzymes studied, except adenosine triphosphatase whereas the pericytes of the vascular granulation tissue are positive for all the enzymes tested 8 peripheral and 2 central oral giant cell granulomas have been studied electron microscopically The ultrastructure of the two varieties is similar, both showing two different types of giant cells Clusters of stromal cells, probably pericytes, are often found, lying in intimate relation with a distance of 150 140 Å between their cell membranes. Though no membrane fusions have been clearly demonstrated the histochemical activities of the giant cells and the pericytes lend support to the assumption, that the pericytes are precursors to the giant cells

The origin of multinuclear giant cells in pathological lesions of the oral cavity has been a matter of discussion during the last decades Based on *light microscopical* studies suggestions have been made that the giant cells of peripheral giant cell granulomas may develop through fusion of mononuclear cells (*Bernier* & *Cahn* 1954, *Adkins et al* 1969b) or by amitotic division of cell nuclei as proposed by *Adkins et al* (1969b) *Cooke* (1952) is convinced that the giant cells of giant cell epulis represent osteoclasts, a cell type supposed to originate from osteoblasts (*Tonna* 1960)

In cell culture, transformation of macrophages into epithelioid cells and their subsequent fusion into giant cells have been documented by *Sutton* & *Weiss* (1966) However, in cultures of giant cell tumours of bone *Schajowicz* (1961) found several areas which might indicate giant cell formation either by amitotic division of the mononuclear cells or by nuclear segmentation

Enzyme histochemical analyses have shown that giant cells of giant cell containing lesions are metabolic active cells (*Barker* & *Klapper* 1961, *Schajowicz* 1961) Furthermore giant cells of both physiological and pathological conditions have shown similar or identical histochemical activities indicating some common histogenetic features (*Pepler* 1958, *Schajowicz* 1961, *Wertheimer* 1967) Because of differences in enzyme activities in giant cells and stromal cells, *Pepler* (1958) found it unlikely that giant

Received 13 ii 73 Accepted 13 ii 73

Requests for reprints should be addressed to Dr *Lis Andersen* Department of Oral Histopathology Royal Dental College 8000 Aarhus C Denmark

cells of osteoclastoma and giant cell epulis are formed by syncytical fusion of stromal cells

In electron microscopical studies of giant cells in peripheral oral giant cell granulomas the finding of irregular nuclei presenting deep indentations has been interpreted as formation of giant cells through amitotic nuclear division of mononuclear cells (*Mazanec & Svejda* 1969 *Drepper et al* 1969) In a work on a giant cell tumour of the long bone, *Hanaoka et al* (1970) observed merging plasma membranes between mononuclear cells, which lead them to infer that cell fusion was occurring This view is shared by *David & Korth* (1959) and *Adkins et al* (1969a) in their studies on oral giant cell granulomas

The aim of the present study has been 1) to report on cellular enzyme activities of oral giant cell granulomas using a new transport medium which facilitates standardized comparative histochemical analyses, 2) to compare the enzyme activity of the giant cells and the stromal cells or what is believed to be the precursor cells, 3) to report on the ultrastructure of oral giant cell granulomas, 4) to compare the functional and morphological data in order to gain insight in the origin and genesis of the giant cells

MATERIAL AND METHODS

Light Microscopical Investigation

Tissue from 8 peripheral and 4 central oral giant cell granulomas was used for enzyme histochemical analyses employing a standardized method Biopsies were taken at different hospitals and were sent by mail to the laboratory in ice cold transport solution Histocon® (Bethlehem Trading AB, Goteborg Sweden *Heyden et al* 1972) and stored in this solution for 24 hours Subsequently the tissue was mounted on brass cryostat chucks Freezing was performed in iso-Pentane (Kebo Goteborg, Sweden) prechilled in liquid nitrogen to approximately −40° C *Sectioning* at 8 microns was carried out in a cryostat at 20° C

Enzyme histochemical evaluation of the metabolic capacities of the cells was made in the light microscope after incubation of tissue sections for the following activities $NADH_2$ diaphorase (EC 1 6 2 1 (?)), *Chayen et al (1969)* $NADPH_2$ diaphorase (EC 1 6 2 3 (?) or EC 1 6 9 9), *Chayen et al* (1969), glucose 6 phosphate dehydrogenase (EC 1 1 1 49), *Altmann* (1968) glutamate dehydrogenase (EC 1 4 1 2), *Chayen et al* (1969) adenosine triphosphatase (EC 3 6 1 3), *Padykula & Herman* (1955), leucine aminopeptidase (EC 3 4 1 1), *Nachlas et al* (1960), acid phosphatases (EC 3 1 3 2, *azo-dye and Gomori lead techniques) Barka & Anderson* (1963)

Controls of possible interfering, non enzymic staining reactions were performed after enzyme inhibitory treatments (10 per cent formalin) of tissue sections and incubation of the sections in media without the respective enzyme substrates Staining for histomorphological purposes was done with the van Gieson technique and haematoxylin and eosin

Electron Microscopical Investigation

Tissue from 8 peripheral and 2 central giant cell granulomas was prefixed in a 6 25 per cent glutaraldehyde solution or in a combined para formaldehyde glutaraldehyde solution (*Karnovsky* 1965) *After postfixation in 2 per cent* osmium tetroxide the tissue was dehydrated and embedded in Epon or Vestopal Semi *thin (1 µm) sections* were cut with glass knives on an LKB Ultrotome III ultramicrotome, and stained with toluidine-blue and paraphenylene diamine (*Establ. Puig et al* 1965) From these sections which were used in a previous study (*Andersen et al* 1973) areas were selected for the ultrastructural study Ultra thin sections (400 800 A) were stained with uranyl magnesium acetate and lead citrate (*Frasca & Parks* 1965 *Reynolds* 1963) and examined in a Philips EM 200 electron microscope

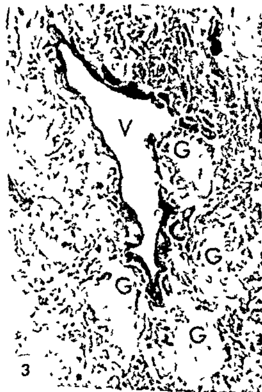
Fig 1 Multinucleated giant cells demonstrating *intense* $NADH_2$*-diaphorase* activity and usually situated in the vicinity of endothelial cells (arrows) *Blood* vessels (*V*) *Nuclei are unstained* × 500

Fig 2 Glutamate dehydrogenase activity in multinucleated giant cells close to bud ends of blood vessels (*B*) *Note a characteristic* loop of a vessel (black arrows) The endothelial lining of some vessels *show a lower but still distinct enzyme* activity (light arrows) Scattered macrophages and a giant cell (G) close to a vessel wall, reveal *intense* staining reactions × 500

Fig 3 Marked ATPase activity visible in the endothelial lining of a large blood vessel (V) The surrounding giant cells (G) are negative × 500

Fig 4 Leucine aminopeptidase activity in *peripheral parts of giant cells* (G) and their immediate extracellular vicinity Endothelial cells (light arrows) of the blood vessels (*V*) are negative × 500


V
V
1

B
B
B
G
2

V
G
G
G
G
3

G
G
V
G
V
G
G
4

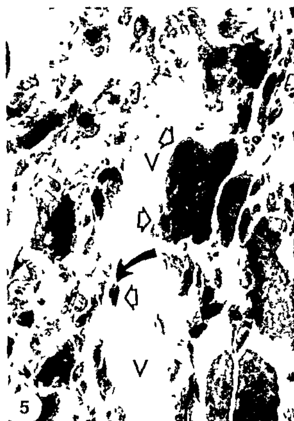




Fig 5 & 6 Intense acid phosphatase activity (azo dye method) in giant cells frequently masking the nuclei Certain mononuclear cells with a pericytic position (black arrows) and sometimes seen in clusters (Fig 6) demonstrate similar enzyme reactions Endothelial cells (light arrows) of the blood vessels (V) are negative × 500

RESULTS

The description of the peripheral and the central giant cell granulomas will be handled together as the two conditions have presented identical cytological features

Enzyme Histochemical Study

The oral giant cell granuloma, both peripheral and central consisted of a highly cellular and vascular granulation tissue containing a varying number of multinucleated giant cells scattered in the tissue The giant cells were often found in close contact with or lining the walls of, the vascular spaces Based on differences in morphology and stain ability of the cytoplasm and the nuclei, the giant cells could be classified into two types The stromal cells varied in shape and stain ability as well as in amount of cytoplasm Spindle-shaped fibroblast like cells with sparse cytoplasm were quite numerous, whereas cells rich in cytoplasm (probably macrophages) were fewer in number

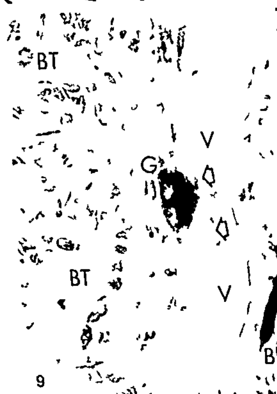
Figs 7 & 8 Haematoxylin and eosin staining of undecalcified bone tissue (BT) of a central giant cell granuloma demonstrating osteoid zones (O) which disappear close to giant cells (Fig 7, G) In areas blood vessels (black arrows) lie in close bone contact (especially Fig 8) × 500

blasts lining the bone tissue (*BT*) *show* weak but still distinct enzyme activity × 500

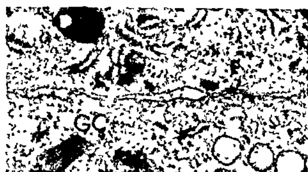
Fig 10 Higher magnification of blood vessels (black arrow) with a local high acid phosphatase activity (azo-dye method) close to bone tissue (BT) To be compared with Fig 8 × *900*

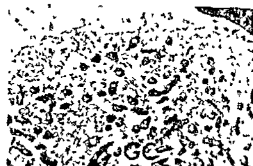

O
BT
BT
O
G
7


O
BT
8


BT
V
G
BT
V
B
9


BT
10





ER
F
ER
11







The enzyme histochemical analyses demonstrated intense *diaphorase* and *glucose-6-phosphate dehydrogenase* activities and moderate *glutamate dehydrogenase* activity in the cytoplasm of the giant cells (Figures 1 and 2) The nuclei were always unstained Similar activities were found perivascularly in cell clusters resembling giant cells Some mono nuclear cells, probably pericytes, and macro phages demonstrated a high activity of these enzymes identical to those found in the giant cells The former cells could easily be dis tinguished from other cellular components which generally showed much weaker enzyme reactions

The *adenosine triphosphatase* (ATPase) activity was high in the endothelial cells of the blood vessels (Figure 3) The giant cells and most other cells outside the vessels ex cept pericytes, however, were negative Osteoblasts could be distinguished from osteoclasts by means of an intense ATPase activity in the former cells

Leucine aminopeptidase activity was found in the cytoplasm of the pericytes, the macro phages, the multinucleated giant cells and in an extracellular zone close to the giant cells (Figure 4) The activity within the giant cells was located exclusively at the periphery just inside the plasma membrane Other cellular components demonstrated a low activity

Reactions of intense *acid phosphatase* activities were registered in certain pericytes, in all macrophages and multinucleated giant cells (Figures 5 and 6), and in perivascularly located cell clusters Within the cells the enzyme activities seemed to be evenly distributed, frequently masking the nuclei Other cell components revealed a very low enzyme activity

Areas of osteogenesis with formation of immature osteoid trabecula as well as areas of bone resorption were found in some granulomas Occasionally an intimate relationship between blood vessels and bony tissue devoid of osteoid substance was present (Figures 7 and 8) Giant cells and cells of the blood vessel walls lying in close proximity to bone trabeculae demonstrated intense acid phosphatase activity (Figures 9 and 10) Cells of other blood vessels showed a low activity

Control sections incubated without substrates, and after treatment with enzyme inhibitor were consistently negative in all histochemical tests

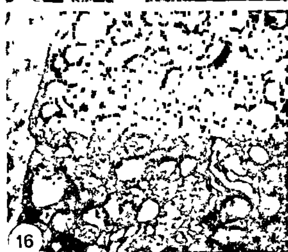
Electron Microscopical Study

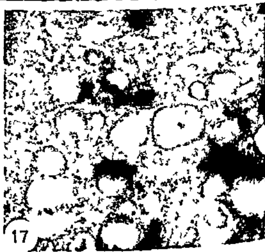
Giant cells The occurrence of two different types of giant cells could be confirmed at the ultrastructural level The most prominent giant cell, type I (Figure 11), had the following cytological characteristics the outline of the cell membrane was irregular with cytoplasmic projections and deep invagina tions Along the plasma membrane a few pinocytic vesicles were found The abundant cytoplasm contained a large number of evenly distributed small rounded or elongated mi tochondria (Figure 11) In most giant cells a well developed rough surfaced endoplasmic reticulum was located mainly at the peri phery of the cells Several small Golgi zones rich in vesicles were often situated juxtanu clearly (Figure 13) Ribosomes and polyribosomes were abundant Membrane bound organelles of varying size belonging to the lyso some group appeared in most giant cells (Figure 14) with a content of fine granular electron dense material and myelin like struc tures Vesicles of varying size and vacuoles devoid of structural components was seen in the cytoplasm (Figure 12) Cytoplasmic mi crotubules (Figure 12) were evenly distri buted and centrioles were present in a few

Fig 11 Part of multinuclear giant cell (GC) and fibroblast (F) The cytoplasm of the giant cell is dominated by mitochondria and small vesicles At the periphery of the giant cell and in the fibro blast a rough surfaced endoplasmic reticulum (ER) is located × 7000

Fig 11, inset Very narrow intercellular space be tween giant cell containing vesicles (GC) and fi broblast (F) × 22000

Figs 12, 13 & 14 Typ cal organelles in the giant cells Mitochondria (M) cytoplasmic microtubules (arrows) vesicles (V), small Golgi apparatus (G) and myeloid figures (MF) × 25500 × 25500 × 14500


16


17

cells Most nuclei were irregular with deep indentations The nuclear membrane presented towards the nucleoplasm a distinct lamina fibrosa of uniform thickness. One or two well-developed nucleoli were found in each nucleus

Giant cells of the type II (Figure 15), much fewer in number, differed from the above described cells in the following way the cytoplasm was much more electron-dense and was dominated by a large number of vacuoles and vesicles (Figures 16 and 17) The rough surfaced endoplasmic reticulum was very distinct due to the differences in density of the cytoplasm and of the matrix of the cisternae These cisternae and the perinuclear space were often dilated to a great extent The number of lysosomes and mitochondria did not differ from that of type I cells The nuclei were shrunken with a very irregular outline and the nucleoplasm was very electron dense

The *stromal cells* were in some areas found in close contact to the giant cells (Figure 11, inset), the distance between the cell membranes being 100 200 A The cytoplasm of the majority of stromal cells contained a well developed rough surfaced endoplasmic reticulum, a moderate number of mitochondria and one or more Golgi zones Lysosomal like figures were present in some cells Fine filaments, 50 80 A in diameter, and polyribosomes were found throughout the cytoplasm The nucleus was similar to those of the giant cells These stromal cells appeared to be of the fibroblastic type Besides these cells, clusters of stromal cells exhibiting a slightly different appearance were seen (Figure 18) Most of these cells had a higher nucleo cytoplasmic ratio, and contained fewer organelles than the fibroblastic type In these clusters the distances between the cell membranes of the individual cells were small, varying from 150 400 A The nuclei were similar to those of the giant cells The clustered cells were separated from the surrounding tissue components by irregularly dispersed lamina densa-like material (Figure 19)

Fig 15 Part of typical multinuclear giant cell type I (GC I) and giant cell type II (GC, II) and fibroblast (F) Nuclei (N) Note the differences in density of the cytoplasm and nuclei in the different types of giant cells and the differences in cytoplasmic content × 7000

Figs 16 & 17 Part of the electron-dense cytoplasm containing numerous vesicles in the type II giant cells. × 14000 × 29000

DISCUSSION

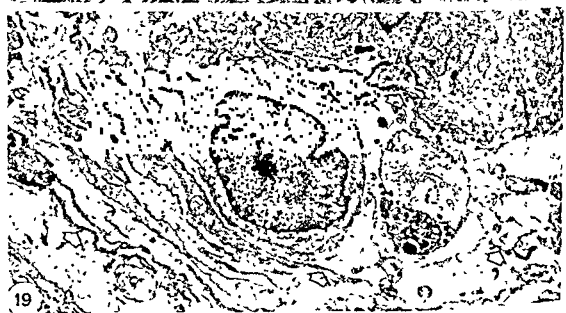
The enzyme histochemical findings in the present study is in agreement with those obtained in most other works using fresh frozen specimens It confirms earlier findings, that the Histocon-transport method does not visibly affect the enzyme reactions in the tissues (*Heyden et al* 1972)

With exception of the ATPase reaction, which was negative, the giant cells exhibited activity for all of the enzymes investigated The high diaphorase and glucose 6 phosphate dehydrogenase activities in the giant cells are identical to those reported by *Fullmer* (1964) and *Wertheimer* (1967) who studied developing bone in the rat and giant cell granulomas, respectively The moderate glutamate dehydrogenase activity and the activity of oxidative enzymes mentioned above corresponds to the biochemical data of isolated osteoclasts presented by *Walker* (1972) The even distribution of a large number of mitochondria in the giant cells parallels the high activity of oxidative enzymes in these cells

The very strong acid phosphatase activity observed in the multinucleated cells is in accordance with the positive reaction in giant cells of oral giant cell granulomas (*Wertheimer* 1967) giant cell tumours of bone (*Kraietski et al* 1970) and isolated osteoclasts (*Walker* 1972) Acid phosphatase are regarded as enzymes derived from lysosomes (*Vorthoff* 1961), and the ultrastructural investigation has confirmed the presence of these structures The diffuse reaction product may be explained by the presence of numerous scattered vesicles, probably of lysosomal origin However the masking of the nuclei seems to indicate that the enzyme reaction products

R

18



are diffusible caused by rupture of the lysosomal membranes during tissue preparation

The positive leucine aminopeptidase reactions in the giant cells are in agreement with the findings of *Wertheimer* (1967) The peripheral localization of the reaction products in the present study might indicate a similar distribution of lysosomal structures, but the ultrastructural investigations does not support this assumption

Wertheimer (1967) has reported on ATPase (EC 3 6 1 4) activity in the giant cells of oral granulomas, in contrast to our results (EC 3 6 1 3), which showed a negative reaction Difference in enzyme batches and techniques employed may account for the divergent findings

The ultrastructure of the giant cells in the present study is to some extent similar to that observed in small series of oral giant cell granulomas (*Gusek* 1958, *David* & *Korth* 1959, *Drepper et al* 1961, *Adkins et al* 1969 a) The presence of two morphological variants of these cells has been described by *Hanaoka et al* (1970) in their studies on giant cell tumours of bone The authors interpreted the giant cells with wide perinuclear spaces and a very electron dense cytoplasm with numerous vacuoles as degenerating cells The degenerating or ageing multinucleated giant cells mentioned in the recently published work on peripheral oral giant cell reparative granuloma by *Sapp* (1972) seem to be identical to those of the type II giant cells described in the present paper According to *Sapp*, a centrosphere with a pair of centrioles usually accompanied each of the scattered nuclei in the giant cells However, we find these structures only infrequently Membrane bounded organelles belonging to the group of lysosomes were a common finding in the giant cells, although *Adkins et al* (1969a) considered these structures to be rare The myeloid figures found in the cytoplasm represent modified secondary lysosomes according to the recent description of similar structures in liver cells (*Hruban et al* 1972)

Engulfed or phagocytized stromal cells in the giant cells as observed by *Sapp* (1972) was not observed in the present material However, the presence of stromal cells with part of the cell membrane lying in close contact with those of the giant cells may represent the initial stages in either such a process or the process of fusion

The ultrastructural finding of stromal cell clusters with intimate intercellular relations makes a distinction between multinucleated giant cells and cell clusters impossible at the light microscopical level It seems likely that the cell clusters are the initial stage in syncytial giant cell formation The cells in these clusters may represent either immature fibroblasts as observed by *Ross* & *Odland* (1968) in their studies on human wound repair or cross sectioned fully differentiated fibroblasts (*Ross* 1968) However, the presence of lamina densa like material surrounding these cells may suggests that they are pericytes which may be enveloped in the same basement membrane as the endothelial cells (*Luft* 1965) Upon stimulation the pericytes increase in number and are often "superimposed one on another" according to *Movat* & *Fernando* (1964) who studied connective tissue from mesentery omentum articular and periarticular tissue of rabbits, and from cheek pouch of hamsters

The ultrastructural findings of the pericytic cell clusters and the enzyme histochemical studies showing closely related activities in the giant cells and the pericytes, may indicate that pericytes are precursors to the giant cells The pericyte clusters may thus represent the initial stages of giant cell formation

---

Fig 18 Cluster of mononuclear stromal cells Note the intimate relationship between the cell membranes (arrows) Red blood cell (B) and fibrin (F) × 3900

Fig 19 In the connective tissue ground substance at the periphery of the cell clusters a great amount of lamina densa like materials are often seen (arrows) × 7000

40*

REFERENCES

Adkins, K F, Martinez, M G & Hartley, M W Ultrastructure of giant cell lesions A peripheral giant cell reparative granuloma Oral Surg *28* 713–723, 1969a

Adkins, K F, Martinez, M G & Robinson, L H Cellular morphology and relationships in giant cell lesions of the jaws Oral Surg *28* 216–222, *1969b*

Altmann, F P The cellular chemistry of certain cytoplasmic oxidative enzymes and their relevance to the metabolism of cancers Ph D Thesis University of London 1968

Andersen, L, Fejerskov, O & Philipsen, H P Oral giant cell granulomas A clinical and histological study of 129 new cases Acta path microbiol Scand Sect A *81* 606–619, 1973

Baker, B L & Klapper, Z F Oxidative enzymes in the foreign body giant cell J Histochem Cytochem *9* 713 714, 1961

Barka, T & Andersen, P J Histochemistry Theory, Practice and Bibliography Hoeber Med Div, Harper & Row New York 1963, p 245

Bernier, J L & Cahn, L R The peripheral giant cell reparative granuloma J Amer dent Ass *49* 141–148, 1954

Chayen, J, Bitensky, L, Butcher, R & Poulter, L A Guide to Practical Histochemistry Oliver & Boyd Edinburgh 1969, pp 200 202 207–208

Cooke, B E D Giant-cell epulis histogenesis and natural history Brit dent J *93* 13–16 1952

David, H & Korth, I Submikroskopische Untersuchungen zur Struktur und formalen Genese der Riesenzellen der Epulis gigantocellulares Frankfurth Z Path *70* 89–97 1959

Drepper, H, Themann H & Wannenmacher, E Untersuchungen der sublichtmikroskopischen Struktur der Riesenzellepulis Dtsch Zahn, Mund u Kieferheilk *35* 1 18 1961

Estable Puig, J F Bauer, W C & Blumberg J M Paraphenylene-diamine staining of osmium fixed plasticembedded tissue for light and phase microscopy J Neuropath exp Neurol *24* 531–535, 1965

Frasca, J M & Parks, V R A routine technique for double staining ultrathin sections using uranyl and lead salts J Cell Biol *25* 157–161 1965

Fullmer, H M Dehydrogenases in developing bone in the rat J Histochem Cytochem *12* 210–214, 1964

Gusek, W Die Feinstruktur der einkernigen Makrophagen und der mehrkernigen Riesenzellen im Fremdenkorpergranulationsgewebe Frankfurt Z Path *69* 429–436 1958

Hanaoka, H, Friedman B & Mack R P Ultrastructure and histogenesis of giant cell tumor of bone. Cancer *25* 1408 1423, 1970

Heyden, G, Arwill, T, Lilja J & Magnusson, B C Chlorhexidine solutions in histological and histochemical techniques J oral Path *1* 12–21, *1972*

Hruban, Z, Slesers, A & Hopkins, E Drug induced and naturally occurring myeloid bodies Lab Invest *27* 62–70, 1972

Karnovsky, M J A formaldehyde glutaraldehyde fixative of high osmolarity for use in electron microscopy J Cell Biol *27* 127A–138A, 1965

Kratevski, N A, Raihklin, N T & Solotiov, J V Histochemical characteristics of giant cell bone tumours Folia histochem cytochem *8* 3–10, *1970*

Luft, J H The ultrastructural basis of capillary permeability In Zweifach B W Grant, L & McCluskey, R T (Eds) The Inflammatory Process Academic Press New York 1965, p 121 159

Mazanec, K & Svejda, J Ultrastructure des cellules geantes de l epulis a cellules geantes et de l osteoclastome Bull Group int Rech sci Stomat *12* 375–388, 1969

Movat, H Z & Fernando N V P The fine structure of the terminal vascular bed IV The venules and their perivascular cells (Pericytes adventitial cells) Exp molec Path *3* 98 114 1964

Nachlas, M M, Morris, B, Rosenblatt, D & Seligman, A M Improvement of the histochemical localization of leucine aminopeptidase with a new substrate, L leucyl 4 methoxy 2 naphtyl amide J biophys. biochem Cytol *7* 261–264, 1960

Novikoff, A B Lysosomes and related particles In Brachet, J & Mirsky A E (Eds) The cell, vol 2 Academic Press New York and London 1961 p 423–488

Padykula H A & Herman E The specificity of the histochemical method for adenosine triphosphatase J Histochem Cytochem *3* 170–183 1955

Pepler W J The histochemistry of giant cell tumours (osteoclastoma and giant cell epulis) J Path & Bact *76* 505 510 1958

Reynolds E S The use of lead citrate at high pH as an electronopaque stain in electron microscopy J Cell Biol *17* 208–212 1963

Ross R The fibroblast and wound repair Biol Rev *43* 51–96, 1968

Ross, R & Odland, G Human wound repair II Inflammatory cells Epithelial Mesenchymal interrelations and Fibrogenesis J Cell Biol *39* 152 168 1968

Sapp, J P Ultrastructure and histogenesis of peripheral giant cell reparative granuloma of the jaws Cancer *30* 1119–1129 1972

Schajowicz F Giant cell tumours of bone (osteoclastoma) J Bone & Jt Surg *43A* 1–28 1961

Sutton, J S & Weiss, L Transformation of monocytes in tissue culture into macrophages epithelioid cells and multinucleated giant cells J Cell Biol *28* 303–332, 1966

Tonna, E A Osteoclasts and the aging skeleton A cytological, cytochemical and autoradiographic study Anat Rec *137* 251 269, 1960

Walker, D G Enzymatic and electron microscopic analyses of isolated osteoclasts Calc. Tiss Res *9* 296–309, 1972

Wertheimer, F W Enzymic histochemistry of giant cell reparative granulomas Oral Surg. *23* 464–469, 1967

Acta path microbiol scand Section A *81, 5,* 630 636, 1973

EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits

1 Concentration of Fluoride in Cortical Bone

JAN ROSENQUIST

The Departments of Oral Surgery and Orthopaedic Surgery, University of Umeå, Sweden

The fluoride content in cortical bone ash of growing rabbits given 0 5 and 10 mg of fluoride per kg weight per day was studied after 2, 4, 7 and 14 weeks The retention was linear The amount of fluoride in the diet, 2 p p m, was not sufficient to maintain the fluoride level in the control group in which there thus was a small decrease After withdrawal of fluoride at practically completed growth (21 weeks) the fluoride content was determined after another 4, 12 and 24 weeks There was no decrease in the fluoride content, suggesting that the fluoride was firmly bound to the bone minerals

It is well established that ingestion of even small amounts of fluoride leads to an accumulation of this element in the hard tissues of the body and that high doses may also cause pronounced morphological changes in bone (cf *Eriksson* 1970) However, little interest has been focussed on the elimination of fluoride from bone tissues and to possible changes in altered bone tissue when fluoride is withdrawn from the diet

Two experiments have been carried out on growing rats *Glock et al* (1941) studied the elimination of fluoride from whole long bones of adult rats which during growth, had been fed a fluoride supplemented diet After withdrawal of the fluoride, about 30 per cent of this incorporated element, calculated as per cent of ash, was eliminated during the first 4 weeks after withdrawal and an additional roughly 30 per cent during another 8 or 10 weeks

Received 12 ii 73 Accepted 12 iii 73

Requests for reprints should be addressed to *Jan Rosenquist,* Department of Oral Surgery, 901 87 S Umeå Sweden

Savchuck & *Armstrong* (1951) found that in fluoride treated growing rats, 10–15 per cent of the fluoride content of the entire humerus was eliminated during the first 40 days after withdrawal of fluoride from the diet The remainder of the skeletal fluoride was not appreciably excreted after another 110 days (still growing at the time of withdrawal of fluoride)

The results of these two studies are somewhat contradictory One reason for this may be that, in the materials studied cancellous and cortical bone which have different metabolical turnover rates were represented in unknown proportions Another could be that the animals in the study by *Savchuck* & *Armstrong* (1951) were still growing at the time of withdrawal of fluoride The effect of with

[illegible]

als is not uncommon in man

The aims of the present investigation were to study

a) the content of fluoride in cortical dia

TABLF 1 *Main Composition of Diets*

	Diet AE*	Diet L§
Dry substance	89 %	89 %
Water	max 11 %	11 %
Protein	20–22 %	30 %
Fat	4 5–5 5 %	2 %
Crude fibre	11–13 %	10 %
Ash	8 %	8 %
Ca	0 7–1 0 %	1 1 %
P	0 5–0 7 %	0 5 %
F†	12 p p m	2 p p m
Salt	0 2–0 3 %	ad libitum
Calories	3 Mcal/kg	2 8 Mcal/kg
Vitamins		
A	15 000 I E/kg	300 I E/day
D_3	1 000 I E/kg	150 I E/day
E	25 mg/kg	2 mg/day
B_1	3 mg/kg	–
B_2	10 mg/kg	–
B_6	4 mg/kg	–
Niacin	50 mg/kg	–
Ca pantothenate	15 mg/kg	–
Folic acid	0 6 mg/kg	–
Choline chloride	400 mg/kg	40 mg/day
Trace elements added Fe, Cu Co, I, Mn, Zn		–

* Analyses from Astra Ewos
§ Own analyses and food tables kindly placed at my disposal by professor S Eriksson, Uppsala
† Own analyses

Diet AE is a pelleted commercial diet from Astra Ewos Diet L is composed of soy meal, hay, minerals and vitamins The components are expressed as per cent of dry weight The vitamins in diet L were ingested daily

physeal bone in growing rabbits given two different known amounts of fluoride daily,

b) the content of fluoride in cortical diaphyseal bone in growing rabbits fed a commercial standard diet, and

c) the elimination of fluoride from the cortical bone after completed growth and withdrawal of fluoride from the diet

This study is one in a series of investigations on the effect of ingestion of fluoride on growing rabbits between 7 and 21 weeks of age and withdrawal after completed growth The findings in this study will in further communications be correlated to parathyroid function alkaline phosphatase activity in serum chemical composition of the organic matrix of cortical bone and the possible reversibility of the morphological bone alterations known as skeletal fluorosis

MATERIAL AND METHODS

243 growing rabbits of both sexes, obtained from the the same farm and of a controlled age of 48–52 days at the beginning of the experiment were fed two different basic diets, called AE and L These were nutritionally equivalent (*Eriksson* 1971) and contained fluoride in unknown salt form Diet AE contained 12 p p m of fluoride and diet L 2 p p m of fluoride (further details of the composition of the diets are given in Table 1) Diet AE was given to groups I 00 and I B while all other groups were fed diet L All animals were given distilled water throughout the experiment and a fluoride supplement as follows

Series I—*the effect of fluoride ingestion in growing rabbits*

Group			
Control	I 0	Diet L	No fluoride supplement
	I 00	Diet AE	No fluoride supplement
	I A	Diet L	0 5 mg of F per kg body weight per day
	I B	Diet AE	10 mg of F^- per day body weight per day
	I B	Diet L	10 mg of F per kg body weight per day

Series II—the effect of withdrawal of fluoride after completed growth

Group			
Control	II 0	Diet L	No fluoride supplement
	II A	Diet L	0 5 mg of F^- per kg body weight per day for 14 weeks
	II B	Diet L	10 mg of F per kg body weight per day for 14 weeks

At a calculated food intake of 60 g per kg body weight and day the control animals received approximately 0 1 mg (group I 0) or 0 7 mg (group I 00) of fluoride per kg body weight and day

Fluoride Administration

Fluoride was administered as NaF in a small volume of distilled water in fixed plastic cups When the cups were emptied the animals were supplied with plain distilled water The experimental animals were supplied with such an amount of water that the cups were empty in the morning

Diets

The food given prior to the experiment at the animal farm was analysed for fluoride, the average

content was 4 p p m (average of three randomly selected samples) The water at the farm contained less than 0 05 p p m of fluoride

Diet AE was a commercial pellet diet (from Astra Ewos, Sodertalje) and diet L consisted of hay, soy meal and a commercial mineral mixture (the composition is given in Table 1) A piece of salt stone was kept in the cages to satisfy the sodium chloride demand

Sample Preparation

The animals were killed by an overdose of sodium pentobarbital The femora and the tibiae were freed from all soft tissues including the periosteum The bone marrow was removed by a sharp blast of air and the bones were rinsed in saline and dried on gauze Samples of cortical bone, obtained as shown in Fig 1, were dehydrated and defatted through three changes of acetone for 72 hours They were then air dried to constant weight The dry pieces were pulverized in a Wiley Laboratory Mill (Intermediate Model) supplied with a 60 mesh sieve

Fluoride Determination

About 50 mg of bone powder was ashed in platinum crucibles at 700° C overnight The fluoride content was measured with a fluoride selective electrode (*Frant* & *Ross jr* 1966) as described by *Singer* & *Armstrong* (1968) in the following modified way The ash was dissolved in 1 ml of 1 0 M HCl Thereafter 3 ml of 1 9 M acetate buffer (pH 5 5) and 5 ml of glass distilled water were added, yielding a pH between 5 0 and 5 2 Within the interval 4 0–6 0 variations in pH do not interfere with the fluoride ion activity in the solution (*Bock* & *Strecker* 1968) Using 1 0 M HCl the ash was easily dissolved and yet the fluoride activity was not affected The 1 9 M acetate buffer had a buffering capacity sufficiently strong to make a pH adjustment unnecessary and an ionic strength permitting omission of the suggested NaCl addition (*Singer* & *Armstrong* 1968)

The fluoride ion activity was measured with a fluoride electrode (Orion 94–09) and a reference electrode (Radiometer K 401) adapted to a Penton top (Metrohm EA 615) and connected to a digital voltmeter (Orion 801) The solution was constantly stirred during equilibration for 10 minutes before being read

Determination of Calcium Phosphorus Ratio

10 mg of ash was dissolved in 1 ml of 1 M HCl and 5 ml of distilled water was added

Calcium was determined on 50 µl samples of the solution added to 8 ml of 0 12 per cent $SrCl_2$ solution (*Willis* 1960) The determinations were made using an atomic absorption spectrophotometer (Unicam SP 90), manipulated according to the instruction book

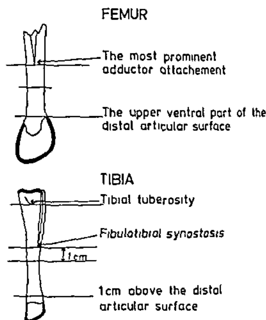


Fig 1 The bones were divided at indicated levels at right angles to their long axis. The middiaphysis of the left femur was divided into a proximal half used for chemical analysis and a distal half used for histochemical studies The left tibia was divided into a proximal and a distal half used for chemical analysis Samples from the right femur and tibia divided in the same way were used for morphological studies

Phosphorus was determined on 100 µl of the sample solution, using the method of *Fiske* & *Subbarow* (1925) as described in the handbook by *Augustinsson* (1966)

The determinations were performed in duplicate

RESULTS

Twenty animals died of infection during the experimental period and 4 by accident The distribution of deaths over the groups was uniform

The remaining animals the numbers of which are given in Table 1 were alert and healthy during the ingestion period and there were no differences in weight gain (Fig 2) After withdrawal of the fluoride supplement, those animals which had received 10 mg of fluoride per kg body weight per day lost appetite seemed to move awkwardly and were irritated when handled However, the

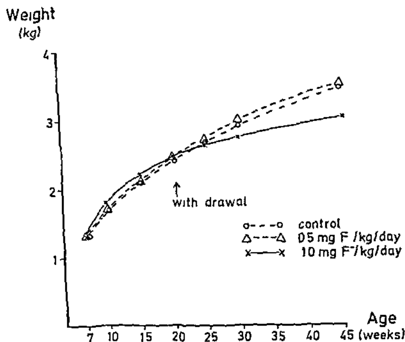


Fig 2 Mean increase of body we ght of control and F⁻ treated rabbits

TABLE 2 *Fluoride Content as Per Cent of Ash in Cortical Bone from the Femur*

a *During fluoride ingestion*

Obs times	0 (7)		2 (9)		4 (11)		7 (14)		14 (21)	
Groups	n		n		n		n		n	
I 0	5	0 030±0 002	6	0 031±0 006	10	0 039±0 012	9	0 027±0 003	9	0 027±0 005
I 00			10	0 039±0 006	10	0 040±0 008	10	0 055±0 005	7	0 082±0 007
I.A			7	0 038±0 006	9	0 046±0 013	10	0 047±0 008	9	0 064+0 004
I B			8	0 131±0 006	9	0 268±0 018	10	0 408±0 042	8	0 734±0 095
I B									7	0 758±0 075

b. *During the period after withdrawal of the fluoride supplement after 14 weeks of ingestion*
Observation times in weeks calculated from the day of withdrawal

Obs times	4 (25)		12 (33)		24 (45)	
Groups	n		n		n	
II 0	6	0 025±0 005	5	0 023±0 004	10	0 023±0 003
II A	6	0 062±0 005	6	0 056±0 003	12	0 059±0 006
II B	8	0 735±0 066	7	0 761±0 060	7	0 687±0 054

M±S D
Observation times are given in weeks and actual age of the animals within brackets.
For details of fluoride administration, see text

TABLE 3 *Fluoride Content as Per Cent of Ash in Cortical Bone from the Proximal Tibia*

a. *During fluoride ingestion*

Obs times	0 (7)		2 (9)		4 (11)		7 (14)		14 (21)	
Groups	n		n		n		n		n	
I 0	5	0 032±0 003	6	0 036±0 005	10	0 042±0 014	9	0 027±0 004	9	0 021±0 003
I 00			10	0 039±0 005	10	0 038±0 006	10	0 060±0 001	7	0 079±0 006
I A			7	0 042±0 006	9	0 045±0 006	10	0 055±0 008	9	0 070±0 007
I B			8	0 153±0 010	9	0 287±0 025	10	0 423±0 031	8	0 762±0 099
I B'									7	0 759±0 097

b *During the period after withdrawal of the fluoride supplement after 14 weeks of ingestion*
Observation times in weeks calculated from the day of withdrawal

Obs times	4 (25)		12 (33)		24 (45)	
Groups	n		n		n	
II 0	6	0 026±0 003	5	0 017±0 008	10	0 019±0 004
II A	6	0 065±0 006	6	0 062±0 003	12	0 062±0 006
II B	8	0 793±0 080	7	0 700±0 056	7	0 720±0 037

M±S D
Observation times are given in weeks and actual age of the animals within brackets
For details of fluoride administration, see text

weight gain in this group did not differ statistically from that of the controls

The water intake varied considerably between animals, the total water intake during one week of observation ranged between 400 and 1000 ml per kg body weight in the control group

Fluoride Content

The fluoride content in the femur and the proximal tibia expressed as per cent of ash is shown in Tables 2 and 3 Only the values for the proximal tibia are given as those for the distal tibia did not differ statistically

During the ingestion period there was an increase in the fluoride content of cortical bone in all groups except I 0 In this group there was a small decrease

There was no difference between the increase of the fluoride content in groups I 00 and I A during the first 7 weeks Nor was there any difference between groups I B and I B' as regards the fluoride content, although the former received a diet containing 12 p p m of fluoride and the latter a diet containing 2 p p m

There was no difference between the femur and the tibia as regards the fluoride content

After withdrawal of the fluoride supplement there was no change in the fluoride content of the ash

Calcium-Phosphorus Ratio

There was no difference between control animals and fluoride treated animals as regards the calcium-phosphorus ratio

DISCUSSION

The observed decrease in the fluoride concentration in the control groups (I 0 and II 0) with increasing age may be due to an increase of the skeletal mass and thus a dilution of the fluoride content Consequently an intake of food containing 2 p p m of fluoride does not seem sufficient to maintain a constant fluoride concentration in cortical bone As the increase in the fluoride content in groups I 00

and I A is small and linear, a concentration of between 2 and 12 p p m of fluoride in the diet would appear to keep the fluoride content of cortical bone at a constant level. Although in an unknown salt form, 12 p p m of fluoride in the diet, which is approximately 0 7 mg per kg body weight per day, gives a fluoride content in the cortical bone which roughly corresponds to that otherwise produced by the same amount of fluoride in an easily resorbable form (e g as NaF)

In the experiments by *Savchuck & Armstrong* (1951) growing rats were given 20 p p m of fluoride over a period of 60 days. These authors reported a rapid initial loss of 10–15 per cent of the fluoride content of the entire humerus after withdrawal of fluoride from the diet. As the fluoride supplement was withdrawn at an age of 87 days viz. the animals were still in the growth period, this loss could be due to normal skeletal turnover in growing individuals. At about 132 days of age, that is when the authors regarded the animals as adult, there was no more loss.

In the investigation by *Glock et al* (1941), however, a rapid loss of fluoride was also observed after withdrawal of a maximal daily fluoride supplement of 7 5 mg of NaF which had been given from 6 weeks of age. At the time of withdrawal, the rats used were adult and the rate of their skeletal turnover would have decreased. However no information was given on either number of animals used or the proportion between cancellous and compact bone in the samples studied. A relatively high amount of cancellous bone in the samples could well have been responsible for the more rapid loss of fluoride reported. However it cannot be excluded that the difference between the findings by *Glock et al* (1941) and those presented in this paper may be due to species variations.

In the present investigation no decrease of the fluoride content was found after withdrawal of the fluoride supplement. It should be noted that only cortical bone was studied and that the animals were adult at the time of withdrawal. This strongly indicates that fluoride is firmly deposited in the bone minerals and in adult animals is only lost during skeletal remodelling. In these animals the cortical bone formed before the fluoride ingestion in many cases was resorbed even after the withdrawal of the fluoride supplement (*Rosenquist*, 1973). If at the same time the skeletal mass decreased, the constant contents of fluoride reported would be due to an increase in the proportion of fluoride containing bone, that is, if the fluoride is mainly found in the bone tissue formed after fluoride supplementation.

From the results in this and other studies it is not possible to say whether fluoride is incorporated solely in the newly formed bone or also in the bone already formed at the beginning of the experiment. In a study by *Weidmann & Weatherell* (1959) an attempt was made to determine the fluoride content of the periosteal and endosteal layers of cortical bone in comparison with the average content. The sampling was, however, done by freehand burring and the paper fails to provide conclusive data. To elucidate the problem, a study on well defined cortical areas with fluorotic changes is presently being undertaken.

The investigation was supported by grants from the *Swedish Medical Council* (24X 3925) and from the *University of Umeå*.

I wish to express my sincere gratitude to *Torsten Eriksson* F D Department of Analytical Chemistry, for valuable advice and help with methodological problems concerning fluoride determination.

REFERENCES

Augustinsson K B (ed) Experimentell biokemi Scandinavian University Books, Kopenhamn 1966 383 384

Bock, R & Strecker, S Direkte elektrometrische Bestimmung des Fluorid Ions Fresenius Z Anal Chem *235* 322–334 1968

Eriksson S Personal communication 1971

Eriksson Y (ed) Fluorides and human health WHO Geneve 1970 p 14–15

Frant M S & Ross jr J W Electrode for sensing fluoride ion activity in solution Science *154* 1553 1966

Glock G E Lowater, F & Murray, M M The

retention and elimination of fluorine in bone Biochem J *35* 1235–1239, 1941
Rosenquist, J Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits 5 Reversibility of skeletal fluorosis after completed growth To be published, *1973*
Savchuck, W B & *Armstrong, W D* Metabolic turnover of fluoride by the skeleton of the rat J biol Chem *193* 575–585, 1951
Singer, L & *Armstrong, W D* Determination of fluoride in bone with the fluoride electrode Analyt Biochem *40* 613–614, 1968
Weatherell, J A & *Weidmann, S M* The skeletal changes of chronic experimental fluorosis J Path Bact *78* 233–241, 1959
Willis, J B The determination of metals in blood serum by atomic absorption spectroscopy I Calcium II Magnesium Spectrochim. Acta *16* 259–278, 1960

Acta path microbiol scand Section A 81, 5, 637-644, 1973

EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits
2 Parathyroid Morphology and Function

JAN ROSENQUIST and LENNART BOQUIST

The Departments of Oral Surgery, Orthopaedic Surgery
and Pathology I, University of Umeå, S 901 87 Umeå, Sweden

Retention of fluoride in the bone apatite increase its crystallinity (Schraer et al 1962 *Zipkin et al* 1962) and decreases its solubility *in vitro* (*McCann & Bullock* 1957, *Singer et al* 1965) It might therefore be expected that the mobilization of calcium would be difficult in individuals with skeletal fluorosis A compensatory increase of parathyroid activity might be necessary to maintain a constant serum calcium level Moreover, the excessive periosteal bone formation seen in skeletal fluorosis increases the demand for calcium

A number of investigations have been carried out in order to explain the increased bone resorption seen in skeletal fluorosis Increased parathyroid activity has been reported after fluoride treatment of rats (*Yates et al* 1964) sheep (*Faccini & Care* 1965) and rabbits (*Faccini* 1967) However there are also reports of unaltered parathyroid activity in human subjects with chronic fluorosis (*Singh et al* 1966) and after fluoride administration to rats (*Raisz & Taves* 1967) The discrepant results may be due to species differences, to differences in the amount of fluoride given or the mode of fluoride administration, or to the methods used for indirect estimation of parathyroid activity

In the present investigation known amounts of fluoride were given to growing rabbits from 7 to 21 weeks of age when skeletal growth had ceased At this time the fluoride supplement was withdrawn and the animals were followed for another 24 weeks

The aim of this investigation was to study the effect of fluoride on the morphology and function of the rabbit parathyroids during fluoride ingestion and after withdrawal of the fluoride The parathyroids were studied qualitatively by light and electron microscopy and semi quantitatively by morphometric determinations on light microscopic sections The serum levels of calcium and magnesium were also estimated

Received 24 iv 73 Accepted 2 vii 73

MATERIAL AND METHODS

Animals and Feeding

Growing rabbits aged 48–52 days at the beginning of the experiment were used In this study the animals were fed a laboratory diet (L) which contained 2 p p m of fluoride in an unknown salt form (for further details of the composition of the diet see *Rosenquist* 1973 a) All animals were given distilled water throughout the experiment and a fluoride supplement as follows

Group	
I 0 and II 0	No fluoride supplement
I B	10 mg of fluoride per kg body weight per day
II B	10 mg of fluoride per kg body weight per day during 14 weeks

NaF was administered in a small volume of distilled water in plastic cups. When the cups were emptied the animals were supplied with an amount of water such that the cups were empty in the morning. The number of animals and observation times are given in Table 1.

Preparation and Analysis of Serum

The animals were fasted for 24 hours before blood sampling. Blood was collected from a marginal ear vein before fluoride administration at day 0, day 4 and thereafter once a week for 14 weeks in groups I 0 and I B. In groups II 0 and II B blood was obtained one week before withdrawal of fluoride from the diet and thereafter once a week for 5 weeks. The blood was allowed to clot, after which serum was removed and stored in glass tubes at –20° C until analysis.

The calcium and magnesium concentrations were determined by atomic absorption spectrophotometry with $SrCl_2$ (*Willis* 1960) after precipitation of proteins with 20 per cent trichloroacetic acid. The absorption was read in a Unicam SP 90 spectrophotometer (Unicam Instr. Ltd, London, England).

Examinations of the Parathyroids

The rabbit has two pairs of parathyroid glands. The superior one lies within the thyroid gland and the inferior one at the lower pole of the thyroid or close to the fascia of the sternothyroid muscle. After killing of the animals by an overdose of sodium pentobarbital the inferior pair was dissected free. The glands, which are pale pink in colour, are usually easy to distinguish from muscles and fat tissue.

Light Microscopy

The glands were fixed for 5 days in a 4 per cent neutral formalin solution. The following embedding procedure was used: 70 per cent alcohol, overnight; 95 per cent alcohol 3 hours, absolute alcohol 1.5 hours, xylene 20 minutes and paraffin (m.p. 50–52° C) 3 hours.

Sections 5 μ thick were cut on a Leitz microtome. The first one hundred sections were discarded. Every 10th section thereafter was stained by Weigert's modification of van Gieson's haematoxylin method to a total number of 20 sections.

Electron Microscopy

The samples were fixed in 2.5 per cent glutaraldehyde in 0.34 M Veronal acetate buffer adjusted to pH 7.4 and postfixed in 1 per cent osmium tetroxide in the same buffer. After rinsing and dehydration the specimens were embedded in Epon 812. The sections were cut in an LKB Ultratome III, stained with uranyl acetate and lead citrate and examined in a Siemens Elmiscope 101.

Morphometric Determinations

Morphometric determinations were made using the point sampling technique as described by *Chalkley* (1943) and *Weibel* (1969).

The nucleo-cytoplasmic ratio was determined using an integrating eyepiece (Zeiss I) equipped with 25 reference points. The ratio was calculated from 20 measurements on 2 different central sections of each gland at a magnification of 800 ×.

The nuclear diameters of the parenchymal cells were estimated with an eyepiece micrometer (Zeiss) at a magnification of 800 ×. The largest diameter (D) and the one perpendicular to it (D) were measured on 25 random nuclei in the central parts of 2 central sections of each gland.

These parameters were estimated on paraffin embedded sections at 7 and 14 weeks of observation during fluoride ingestion and at 4, 12 and 24 weeks after withdrawal of the fluoride supplement.

The nuclear diameters of the parenchymal cells were also estimated on Epon embedded sections. These sections were toluidine blue stained and 1 μ thick. The determinations were made at 14 weeks of observation, 25 random nuclei. On these sections, *the nucleo-cytoplasmic ratio* was also calculated using an eyepiece with 121 reference points within a square. The *cell density* was calculated as the number of cell nuclei within the square. The values were obtained from ten measurements of each section at a magnification of 1 000×.

The values obtained at the morphometric analysis were not adjusted with regard to *Holmes* effect.

Statistical Determinations

D[illegible]

the standard deviation of the single observation using the formula $s_e = \sqrt{\frac{\Sigma d^2}{2n}}$ where d denotes the difference between respective pairs of determinations and n the number of double determinations performed. The degree of significance was tested with Student's t test.

RESULTS

Calcium and Magnesium in Serum

The serum calcium in the control group ranged from 7.11 to 7.67 mEq/l and in the fluoride treated groups from 7.06 to 7.55 mEq/l. The ranges for serum magnesium were 2.08–2.54 and 2.02–2.66 mEq/l, respectively. There were no significant differences

between the groups with regard to serum levels of calcium and magnesium

Light Microscopy

The parathyroid glands from the animals in the various groups were essentially similar They were rounded or oval and surrounded by a thin capsule of connective tissue The stroma was delicate and moderately vascularized Light and dark chief cells made up the bulk of the parenchyma These cells possessed a moderately large rounded nucleus with a delicate chromatin pattern and a rather distinct nucleolus The cytoplasm was moderately abundant and either light or dark A few transitional water clear cells and transitional oxyphil cells were occasionally encountered The parenchymal cells formed solid sheets with only a slight tendency to follicular arrangement No adenomatous transformation was observed

Electron Microscopy

The light microscopic observations were verified by electron microscopy The fine structural changes were consistent with a moderately high activity in the parathyroid parenchyma No obvious structural differences between the animal groups were observed

The chief cells possessed oval or slightly irregular nuclei with finely dispersed chromatin and rather distinct nucleoli The electron density of the cytoplasm varied Free ribosomes were scattered in the ground substance The number and size of the mitochondria varied Most mitochondria were rather small oval or elongated and possessed distinct transverse cristae Small intramitochondrial granules were occasionally seen but there were no large intramitochondrial granules The endoplasmic reticulum was rough surfaced and either lamellar or vesicular (Fig 1) Most often the endoplasmic reticulum was rather inconspicuous The Golgi complexes were often small The cytoplasmic particles interpreted as secretory granules varied in frequency size electron density and distribution (Figs 2 and 3) Most granules possessed a rounded core of high electron density with a closely applied membrane No obvious signs

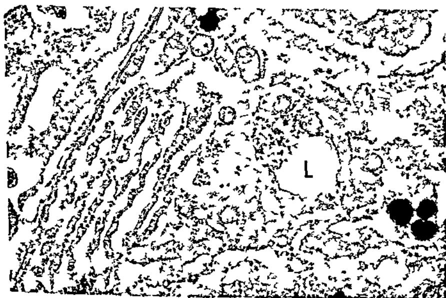


Fig 1 Electron micrograph of a parathyroid gland from a 45 weeks old fluoride treated rabbit showing chief cells with prominent rough-surfaced endoplasmic reticulum mainly of lamellar type dense cytoplasmic particles a lipoid body (L) and straight cell membranes × 10 000

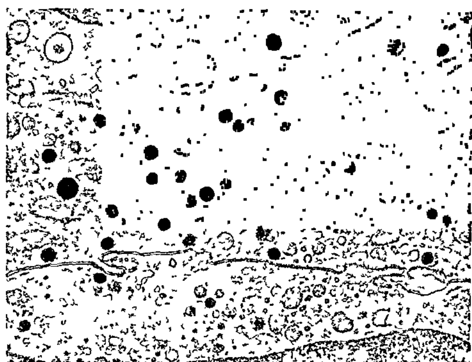


Fig 2 Parathyroid chief cells from a 21 weeks old fluoridetreated rabbit showing secretory granules of varying size and density, and cell membranes with occasional interdigitations × 13 000



Fig 3 Parathyroid chief cells from a 14 weeks old control rabbit showing secretory granules Golgi complexes (G), and cell membranes with a few indentations ×13 000

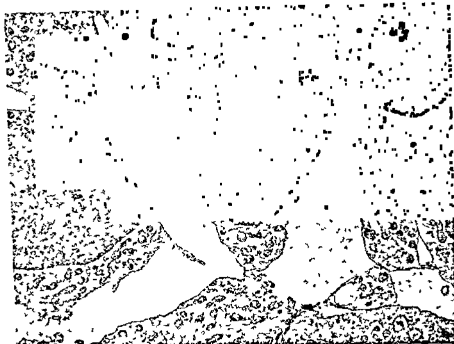


Fig 4 Parathyroid gland from a 45 weeks old fluoride treated rabbit showing chief cells with occasional microvilli and dilated intercellular spaces containing amorphous masses × 5 000

of emicytosis (exocytosis of secretory granules), were observed Lysosomal bodies, fat droplets, accumulations of glycogen and cilia were occasionally found in the chief cells No annulate lamellae were identified Dilated intercellular spaces were seen in a few glands (Fig 4) These spaces were either electron-lucent or contained amorphous masses of low density The cell membranes were usually straight with occasional indentations and a few microvilli The oxyphil cells were characterized by an abundance of rather large mitochondria No essential fine structural changes were seen in the transitional cell types

Morphometric Studies

The results of the measurements made on paraffin embedded sections are shown in Table 1 and those made on Epon embedded sections in Table 2 The nucleo-cytoplasmic ratio is expressed as per cent of nuclear ' hits" in the microscopic field and the nuclear size in μ^2 The cellular density is expressed as number of cell nuclei per μ^2

There were no statistical differences between the groups when tested with Student's t test

DISCUSSION

The best measure of parathyroid function would probably be determination of the parathormone level in blood Immunological methods for determination of this level are available for some species, but not for some of the most common laboratory animals, *e g* rats and rabbits Inasmuch as the nuclear size and nucleo cytoplasmic ratio are known to be related to cellular activity, these parameters were used in the present study for the estimation of parathyroid activity. It cannot be excluded that increased production of parathormone might occur in the absence of morphological alterations in the parathyroid parenchymal cells However, a supplement of 10 mg of fluoride per kg body weight per day to growing rabbits causes excessive bone resorption (*Rosenquist* 1973b), a prominent

TABLE 1 *Morphometric Determinations on Paraffin Embedded Sections*

Group	Obs time	n	Nuclear size/μ^2	n	Nuclear hits per cent
a *During fluoride ingestion*					
I 0	7 (14)	5	14 5 ± 1 1	5	29 2 ± 5 2
I B	7 (14)	7	13 7 ± 1 3	7	28 1 ± 2 7
I 0	14 (21)	5	14 4 ± 1 6	5	29 3 ± 1 0
I B	14 (21)	8	12 6 ± 1 4	8	26 5 ± 2 9
b *During the period following withdrawal of the fluoride supplement after 14 weeks of ingestion* Observation times in weeks calculated from the time of withdrawal					
II 0	4 (25)	5	12 5 ± 1 7	5	29 4 ± 3 7
II B	4 (25)	5	13 2 ± 1 4	5	29 7 ± 2 6
II 0	12 (33)	5	12 7 ± 0 5	5	24 3 ± 3 0
II B	12 (33)	5	13 5 ± 1 2	5	27 4 ± 2 8
II 0	24 (45)	5	12 0 ± 1 0	5	25 6 ± 2 5
II B	24 (45)	5	12 9 ± 0 7	5	27 5 ± 1 8

Observation times are given in weeks and the actual age of the animals within brackets The experimental animals (I B, II B) were given 10 mg of fluoride per kg body weight per day M ± S D
The precision of the determinations (s_e) was for nuclear size 2 6 per cent and nuclear hits 2 3 per cent

TABLE 2 *Morphometric Determinations on Epon Embedded Sections at 14 Weeks of Observation*

	Controls n = 5	Experimentals n = 5	
Nuclear hits per cent	18 1 ± 0 8	18 9 ± 0 9	s_e = 1 1*
Nuclear size μ^2	11 9 ± 0 2	11 8 ± 0 4	s_e = 0 8*
Cell density nuclei/μ^2	0 008 ± 0 0007	0 008 ± 0 0004	s_e = 0 0005*

The experimental animals were given 10 mg of fluoride per kg body weight per day M ± S D
* s_e *denotes the precision of the determination*

feature of skeletal fluorosis If this bone resorption would be the result mainly of parathormone activity, it is not likely that this should occur without associated changes in the parathyroid morphology

The excessive bone resorption in skeletal fluorosis has been supposed to be due to secondary hyperparathyroidism (c f *Faccini* 1969) However our results do not support the opinion that secondary hyperparathyroidism develops The light and electron microscopic examinations disclosed various degrees of activity in the parathyroid parenchymal cells but no clear difference between the control and experimental animals was found Nor were any differences between the groups observed with respect to nuclear size or nucleo-cytoplasmic ratio in the parenchymal cells, parameters known to be related to the cellular activity However, there was considerable individual variation in these respects Thus neither the qualitative nor the semiquantitative morphologic studies provided any evidence of hyperparathyroidism in the fluoride treated animals

Our results are in agreement with the findings by *Singh et al* (1966) and *Raisz* & *Taves* (1967) *Singh et al* (l c) found no evidence of secondary hyperparathyroidism in human subjects with chronic skeletal fluorosis if studied with a calcium deprivation test In the study of *Raisz* & *Taves* (1967) the uptake of the non-metabolizable amino acid alpha amino-isobutyric acid was not in-

creased in fluoride treated growing rats, as would have been expected if the parathyroid activity had been increased

In the study of *Yates et al* (1966) increased osteoclasia was observed in the femurs of growing rats after peritoneal lavage with fluoride containing solutions (0 036 and 0 072 g NaF per l) This increase was only found in rats with intact parathyroids and not in thyreo-parathyroidectomized animals It is possible that peritoneal lavage causes sudden hypocalcaemia since calcium probably will be rapidly deposited in the bone minerals together with the fluoride ions Increased parathyroid activity would then be necessary to restore and maintain calcium homeostasis Hypocalcaemia is a symptom of acute fluoride poisoning (*Waldbott* 1963) and it cannot be excluded that acute fluoride poisoning is studied by the peritoneal lavage technique If so, the results obtained would not be relevant for chronic fluorosis

Faccini & Care (1965) have reported signs of increased activity in the parathyroids of lambs given fluoride containing water (200 p p m of NaF) The fine structural changes were slight after one week of fluoride administration, but marked after one month An immuno assay on one pair of twin lambs disclosed a five fold increase of the serum level of parathormone, but there was no evidence of increased resorption of the long bones which is a common feature in skeletal fluorosis Nonetheless, the authors state that their results point to the parathyroid glands being overactive in severe skeletal fluorosis"

In a similar study on growing rabbits given drinking water containing 200 p p m of NaF for 8 weeks, *Faccini* (1967) reported ultrastructural changes consistent with increased activity of the parathyroid glands It is difficult to explain these findings in the light of our results Faccini s idea of the development of secondary hyperparathyroidism seems, however to be based upon the opinion that fluoride containing bones are resistant to resorption (*Faccini*, 1969) In the morphologic study of long bones from the same animals as those examined here, resorption was observed even in the bone formed during fluoride ingestion (*Rosenquist* 1973b).

As we could not find any clear evidence of increased parathyroid activity, it would seem that bone resorption in skeletal fluorosis is, at least partly, controlled by mechanisms outside the parathyroid glands Resorption was often seen in areas with excessive new bone formation or remodelling of bone, which suggests that local factors in the bones may play a role in the bone resorption.

This investigation was supported by grants from the *Swedish Medical Research Council* (project 24X-3925) and the *University of Umeå*

REFERENCES

Chalkley, H W Method for the quantitative morphologic analysis of tissues J nat. Cancer Inst 4 47–53, 1943

Faccini, J M Inhibition of bone resorption in the rabbit by fluoride Nature 214 1269–1271, 1967

Faccini, J M Fluoride and bone Calcif Tiss Res 3 1–16 1969

Faccini, J M & Care, T D Effect of sodium fluoride on the ultrastructure of the parathyroid glands of the sheep Nature 207 1399–1401, 1965

McCann, H G & Bullock, F A The effect of fluoride ingestion on the composition and solubility of mineralized tissues of the rat J dent Res 36 391 398 1957

Raisz, L G & Taves, D R The effect of fluoride on parathyroid function and responsiveness in the rat. Calcif Tiss. Res 1 219–228 1967

Rosenquist, J Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits 1 Concentration of fluoride in cortical bone Sect A 81 630–636, 1973

Rosenquist J Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits 5 Reversibility of skeletal fluorosis after completed growth To be published 1973

Schraer H, Posner, A S, Schraer, R & Zipkin, I Effect of fluoride on bone 'crystallinity in the growing rat Biochem. biophys Acta 64 565–567 1962

Singer L Dale, M D & Armstrong, W D Effects of high fluoride intake on utilization of dietary calcium and solubility of calcified tissues J dent Res 44 582–586, 1965

Singh, A Singh B M, Singh, I D, Jolly, S S & Malhotra K C Parathyroid function in en-

41*

demic fluorosis Indian J med Res *54* 591–597, 1966

Waldbott, G L Acute fluoride intoxication Acta med scand suppl 400, s 23, 1963

Weibel, E R Stereological principles for morphometry in electron microscopic cytology Int Rev Cytol *26* 235–302, 1969

Willis, J B The determination of metals in blood serum by atomic absorption spectroscopy Spectroch Acta *16* 259–278, 1960

Yates, C, Doty, S & *Talmage, R V* Effects of sodium fluoride on calcium homeostasis. Proc. Soc exp Biol *115* 1103–1109, 1964

Zipkin, I Posner, A S & *Eanes, E D* The effect of fluoride on the X ray differaction pattern of the apatite of human bone Biochim biophys. Acta *59* 255–258, 1962

Acta path. microbiol scand Section A 81, 5, 645-650, 1973

EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits
4 Serum Alkaline Phosphatase Isozymes

JAN ROSENQUIST

The Departments of Medical Genetics, Oral Surgery and Orthopaedic Surgery, University of Umeå, Sweden

Serum alkaline phosphatase isozymes were studied in growing (series I and III) and adult (series II) rabbits given known amounts of fluoride Any quantitative or isozyme differences between normal and fluoride treated animals were not observed Electrophoretic studies indicate that the liver is the major contributor to the serum alkaline phosphatase isozymes in the rabbit.

Serum alkaline phosphatase was long thought to be of osseous origin The development of new techniques for electrophoretic separation made it possible to demonstrate the presence of multiple molecular forms (isozymes) of alkaline phosphatase in various tissues (cf *Fishman & Ghosh* 1967) In cases of osteoblastic bone diseases, an increase of bone alkaline phosphatase in serum has been demonstrated (*Taswell & Jeffers* 1963, *Yong* 1967)

The effect of fluoride administration on the alkaline phosphatase activity in serum has been studied in a number of investigations The fluoride induced excessive bone changes and the increased alkaline phosphatase activity in fluorotic bone tissue (*Miller & Shupe* 1962) were thought to be reflected in an augmented serum alkaline phosphatase activity

Weidmann et al (1959) found an increase of serum alkaline phosphatase activity after fluoride administration to halfgrown and adult rabbits In a similar study on rats they observed no effect on the serum alkaline phosphatase activity On the contrary, *Riekstniece et al* (1965) reported a decrease in alkaline phosphatase activity in the serum of fluoride treated rats as determined by starch gel electrophoresis

Srikantia & Siddiqui (1965) and *Singh et al* (1966) reported an increased activity of serum alkaline phosphatase in persons with chronic fluorosis In previous investigations, no attempts were made to distinguish between different alkaline phosphatase isozymes or to determine their tissue origin The animals were studied at one observation time only and the fluoride was given in uncontrolled amounts

This communication is a part of an investigation on the effect of ingestion of fluoride on growing rabbits between 7 and 21 weeks of age and withdrawal at completed growth

The effect of fluoride on the serum alkaline phosphatase isozymes of growing rabbits given known amounts of fluoride has been investigated in the present study

Received 12 ii 73 Accepted 12 iii 73

Requests for reprints should be addressed to *Jan Rosenquist* Department of Oral Surgery 901 87 S Umeå Sweden

MATERIAL AND METHODS

Experimental Animals and Feeding

The investigation was carried out in three separate series called I, II and III. Ninety four growing rabbits of both sexes and at an age of 40 50 days were fed three different diets, AE, L and C. These were nutritionally equivalent (*Eriksson* 1971) and contained fluoride in unknown salt form. Diet AE contained 12 p p m of fluoride, diet C less than 1 p p m and diet L 2 p p m. Diet C was a cariogenic diet of 50 per cent sucrose, 18 per cent skimmed milk powder, 11 per cent potato flour, 15 per cent whole wheat meal, 5 per cent lucerne meal and 1 per cent yeast. Details concerning the composition of diets AE and L are given in a previous paper (*Rosenquist* 1973 a). The animals in series I and II were from another farm than those in series III. Diet AE was given to series I, diet L to series II and diet C to series III. All animals were given distilled water throughout the experiment and a fluoride supplement as follows

Series I

Control	I 00	No fluoride supplement
	I B	10 mg of fluoride per kg body weight per day

Series II

Control	II 0	No fluoride supplement
	II B	10 mg of fluoride per kg body weight per day. After 14 weeks the fluoride was withdrawn

Series III

Control	III 0	No fluoride supplement
	III A	0 2 mg of fluoride per kg body weight per day
	III B	10 mg of fluoride per kg body weight per day

Fluoride Administration

NaF was administered in a small volume of distilled water in fixed plastic cups. When the cups were emptied the animals were supplied with distilled water. The experimental animals were supplied with such an amount of water that the cups were empty in the morning

Sample Preparation

The animals were fasted for 24 hours before the collection of serum samples in order to avoid dietary effects on the serum alkaline phosphatase activity (cf *Langman et al* 1966)

About 2 ml of blood was collected from a marginal ear vein in *series I* immediately before fluoride administration (day 0) after 4 days and thereafter once a week for 14 weeks. In *series III*, blood was collected on days 0, 4, 8, 28 and 56. The number of animals in series I is given in Table 1. In series III, the number of animals was diminished after 14 and 28 days when some randomly selected animals were killed for other purposes (Table 2). In *series II*, blood was collected from 10 animals in each group one week before withdrawal of fluoride from the diet and thereafter once a week for 5 weeks. The blood samples were allowed to clot and the serum was removed and stored in glass tubes at 20° C until enzyme analysis

Tissue extracts of liver, bone and intestine were prepared in order to compare the isozymes of these tissues with the serum isozymes

The liver and intestinal tissues were cut into

to increase the extraction (*Morton* 1954) and centrifuged for 15 minutes. The supernatant water phase was collected and stored at –20° C. The bone samples were sectioned in thin slices in a freezing microtome. The slices were suspended in saline and centrifuged for 15 minutes. The supernatant water phase was stored at –20° C

Quantitative Determination of Serum Alkaline Phosphatase

Alkaline phosphatase activity was determined in 0 5 M 2 amino- 2 methyl 1 propanol (AMP) buffer at pH 10 8 with di sodium alpha naphthyl phosphate as a substrate (cf *Beckman* 1970). The absorption of liberated alpha naphthol was recorded in a Zeiss or a Unicam SP 500 spectrophotometer at 335 μ (*Moss* 1966). The enzyme activity was expressed as micromoles of alpha naphtol released per 1 h per ml of serum

After *in vitro* addition of fluoride to final concentrations of 1, 0 5 0 25 0 1 and 0 05 p p m in serum samples the phosphatase activity was determined as described above. Saline was used in the control sera and determinations were made in triplicate

Electrophoresis

Alkaline phosphatase isozymes in serum and

buffer using di sodium alpha naphthyl phosphate as a substrate and Blue RR salt as a dye coupler

RESULTS

In series I 5 animals died of infection and in series III 6 animals. The remaining animals

remained healthy throughout the experiment except some animals in group II B which lost appetite after withdrawal of the fluoride supplement The weight gain in this group did not differ from that of the controls

During the experiment, excessne bone resorption and new bone formation occurred in the animals given a supplement of 10 mg of fluoride per kg body weight per day The reversibility of these changes will be reported in another paper (*Rosenquist* 1973 b)

Quantitative Analysis

In series I there was a decrease of the serum alkaline phosphatase activity with in ereasing age (Table 1) After roughly 2 weeks, the activity was stabilized with minor fluctuations There was no difference between treated and untreated animals

There was no difference between controls and fluoride treated animals in series II and there was no change in the serum alkaline phosphatase activity after withdrawal of fluoride

Table 2 shows the results obtained in series III The initial activities were very similar in all groups and in all groups the activity increased with increasing age After 14 days, the serum alkaline phosphatase activity was significantly lower in group III B compared with the control group, but in general, no certain effect of fluoride on the alkaline phosphatase activity could be demonstrated

In vitro addition of fluoride to final con centrations of 1 0 5, 0 25, 0 1 and 0 05 p p m in serum samples did not affect the alkaline phosphatase activity

TABLE 1 *Experimental Series I Serum Alkaline Phosphatase Activity Expressed as Mirro Moles of Alpha Naphtol Liberated per ml of Serum per Hour M±SE*

Obs time		Groups			
		n	I 00	n	I B
Day	0	7	9 31±0 59	8	11 65±0 64
Day	4	7	7 99±0 74	8	9 48±0 59
Week	1	7	7 86±0 87	8	8 67±0 48
	2	7	7 77±0 60	8	7 75±0 31
	3	7	7 16±0 55	7*	7 95±0 49
	4	7	7 12±0 54	8	8 36±0 73
	5	7	6 95±0 40	8	7 73±0 75
	7	7	6 70±0 56	6§	7 51±0 76
	10	7	7 69±0 84	8	8 06±0 60
	11	7	7 82±0 41	8	7 98±0 53
	12	6*	7 49±0 84	7*	7 90±0 88
	13	7	7 05±0 63	8	7 62±0 72
	14	7	7 29±0 81	8	7 42±0 59

* one sample lost
§ two samples lost

Electrophoretic Studies

Three different zones of serum alkaline phosphatase activity were demonstrated by means of electrophoresis in poly-acrylamide gels The zones were called A, B and C in decreasing order of mobility towards the anode (Fig 1) The C zone was present in all serum samples except two The B zone was found only in a few samples in series III The A zone was found in 60 per cent of all serum

TABLE 2 *Experimental Series III Serum Alkaline Phosphatase Activity Expressed as Micro Moles of Alpha Naphthol Liberated per ml of Serum per Hour M± SE*

Obs time Days	Control group		Fluoride treated groups			
	n	III·0	n	III A	n	III B
0	15	7 63±0 65	15	7 70±0 56	18	7 64±0 53
4	15	8 82±0 72	15	9 90±1 09	18	10 72±0 61
8	15	14 89±1 65	15	12 27±1 43	17*	11 19±0 80
14	15	19 35±1 98	15	19 87±2 49	18	13 58±1 13
28	9	25 53±3 69	9	20 30±2 13	12	19 65±2 58
56	4	20 15±3 08	5	24 58±3 75	5	18 80±1 58

* one sample lost



Fig 1 Three different zones of serum alkaline phosphatase activity demonstrated by means of electrophoresis in a polyacrylamide gel The zones are called A B and C in decreasing order of mobility towards the anode (+) Di sodium alpha naphthyl phosphate is used as a substrate and Blue RR salt as a dye coupler

samples in series III, but was not found in series I or II

A comparison with extracts of intestine liver and bone showed that the A and C zones in serum coincided electrophoretically with two zones in liver extracts The B zone had the same mobility as the intestinal alkaline phosphatase component Bone alkaline phosphatase had an electrophoretic mobility corresponding to that of the serum alkaline phosphatase C (Fig 2) The two zones in the liver extracts corresponding to the A and C zones in serum were found in all liver extracts studied, even in animals with only the C zone in their sera

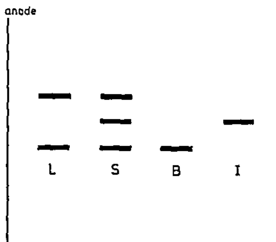


Fig 2 Schematic figure showing the electrophoretic mobility of alkaline phosphatase isozymes from liver (L), serum (S), bone (B) and intestine (I)

The intensities of the A and C zones in series III were scored visually according to the following system The A enzyme, 0 = no detectable zone, 1 = weak zone and 2 =

TABLE 3 a *Distribution of A Scores by Time and Experimental Groups in Experimental Series III*

	Score	Day 0	4	8	14	28	56
Control III 0	0	14	4	2	1	0	0
	1	0	9	6	3	4	2
	2	1	2	7	11	5	2
Group III A	0	9	4	1	0	0	0
	1	6	8	6	4	1	0
	2	0	3	8	11	8	4
Group III B	0	10	1	0	0	0	0
	1	8	14	9*	6	5	1
	2	0	3	8	12	7	4

* one sample lost

TABLE 3 b *Distribution of C Scores by Time and Experimental Groups in Experimental Series III*

	Score	Day 0	4	8	14	28	56
Control III 0	1	4	4	4	1	4	0
	2	11	11	11	14	5	4
Group III A	1	4	3	5	0	1	0
	2	11	12	10	15	8	4
Group III B	1	8	5	4*	1	1	0
	2	10	13	13	17	11	5

* one sample lost

TABLE 4 *Relationship between A and C Scores in Experimental Series III*

A	C 1	2	Total A
0	19	27	46
1	24	68	92
2	6	91	97
Total C	49	186	235

TABLE 5 *Experimental Series III Relationship between Alkaline Phosphatase Scores and Serum Alkaline Phosphatase Activity Expressed as Micro Moles of Alpha Naphthol Liberated per ml of Serum per Hour M ± SE*

A score	C score 1	2	Total A
0	6 55±0 47	7 87±0 48	7 33±0 34
1	9.57±0 81	10 86±0 48	10 53±0 42
2	13 90±0 86	20 00±0 74	19 60+0 74
Total C	8 93±0 70	14 90±0 55	

strongly staining zone For the C enzyme, 1 = weak zone (or absence of zone) and 2 = strongly staining zone

The intensities of the alkaline phosphatase correlation between the A and C isozymes (Table 4) The differences between each A and C score class with respect to the serum alkaline phosphatase activity measured by the quantitative method (Table 5) were highly significant ($p < 0.001$)*

DISCUSSION

In one experimental series (I) in this study, the alkaline phosphatase activity was found to decrease with increasing age In this series, isozyme A occurred infrequently In another series (III), the enzyme activity increased with increasing age The increase in the two alkaline phosphatase isozymes A and C was simultaneous The difference between the animals in series I and III, which came from different farms, may be due to genetic factors, but further investigations are needed to elucidate this matter

Except for one observation there was no significant difference in alkaline phosphatase activity of control animals and fluoride treated animals

The results in this study differ from those reported by *Weidmann et al* (1959) who found an increase in the alkaline phosphatase activity in growing and adult rabbits after fluoride ingestion Their results were, however, based upon a single observation from each animal, the exact age of which was unknown The number of animals was small and no information was given about the method used for phosphatase determinations

Riekstniece et al (1965) reported a decrease in the serum alkaline phosphatase activity in fluoride treated growing rats and explained it as being due to lack of magnesium ions for activation of the enzyme The rats were given three different levels of fluoride in the drinking water and three observation times were used A total of 120 rats were used for the experiment, but only 55 sera were studied by electrophoresis No information was given about the number of analysed sera from the different groups and, as their results were based upon a single serum sample from each rat, they must be interpreted with caution The decrease could be due to e g

* Tested with Student s t test

increasing age, which cannot be excluded on the basis of the data presented Furthermore, it has been found in a recent study that the magnesium content in serum was not affected by fluoride ingestion (*Rosenquist & Boquist* 1973) and addition of fluoride *in vitro* in concentrations reported to be found in serum samples (*Armstrong & Singer* 1970) did not change the alkaline phosphatase activity

In the studies by *Srikantia & Siddiqui* (1965) and *Singh et al* (1966) according to which the serum alkaline phosphatase activity was increased, care had not been taken to safeguard that the control individuals were in the same states of health as the patients with skeletal fluorosis Their results could easily be explained as sequelae of an affected state of health, perhaps changes in the liver, or they could simply be due to genetic factors

In this study there was no distinct difference in isozyme patterns of liver and bone extracts However, in series III there was a simultaneous increase in the scores of isozymes A and C with a concomitant increase of the total serum alkaline phosphatase activity As isozymes A and C were always found in the liver extracts whereas A was never observed in bone extracts, the C isozyme in serum is probably of liver origin

Taken together, the present results of isozyme studies and quantitative determinations indicate that the liver is the major source of serum alkaline phosphatase in the rabbit

Excessive changes in bone morphology with an increase in bone formation and bone re sorption were noted in the fluoride treated animals However they were not reflected in an alteration of the alkaline phosphatase activity in the rabbit serum

This investigation was supported by grants from the *University of Umeå*

I wish to thank Professor *Lars Beckman* Department of Medical Genetics for helpful advice and valuable criticism

REFERENCES

Armstrong, W D & Singer L Distribution in body fluids and soft tissues In Eriksson Y (Ed) Fluorides and human health WHO, Geneva 1970, p 94–104

Beckman, G Placental alkaline phosphatase relation between phenotype and enzyme activity Hum Hered 20 74–80, 1970

Eriksson S Personal communication 1971

Fishman, W H & Ghosh, N K Isoenzymes of human alkaline phosphatase Advanc clin Chem *10* 255, 1967

Langman, M J S, Leuthold, E, Robson, E B, Harris, J, Luffman, J E & Harris, H Influence of diet on the 'intestinal' component of serum alkaline phosphatase in people of different ABO groups and secretor status Nature *212* 41–43, 1966

Miller, G W & Shupe, J L Alkaline bone phosphatase activity as related to fluoride ingestion by dairy cattle Amer J vet Res *23* 24–31, 1962

Morton, R K The purification of alkaline phosphatase of animal tissues Biochem J *57* 595–603, 1954

Moss D W A note on the spectrophotometric estimation of alkaline phosphatase activity Enzymologica *31* 193–202 1966

Poulik, M A Starch gel electrophoresis in a discontinuous system of buffers Nature *180* 1411 1957

Riekstniece E, Myers, H M & Glass, L E In vivo effects of sodium fluoride on serum proteins and enzymes as studied with starch gel electrophoresis Arch oral Biol *10* 107 117 1965

Rosenquist, J Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits 1 Concentration of fluoride in cortical bone Sect A *81* 630–636 1973a

Rosenquist, J Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits. 5 Reversibility of skeletal fluorosis after completed growth To be published 1973 b

Rosenquist J & Boquist L Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits 2 Parathyroid morphology and activity Sect A *81* 637 644, 1973

Singh A Singh, B M, Singh J D, Jolly S S & Malhotra, K C Parathyroid function in endemic fluorosis Indian J med Res *54* 591–597 1966

Srikantia S G & Siddiqui A H Metabolic studies in skeletal fluorosis Clin Sci *28* 477–485 1965

Taswell H F & Jeffers D M Isoenzymes of serum alkaline phosphatase in hepatobiliary and skeletal diseases Amer J clin Path *40* 349–356, 1963

Weidmann S M Weatherell J A & Whitehead R G The effects of fluoride on the chemical composition and calcification of bone J Path. Bact *78* 435–445 1959

Yong J M Origins of serum alkaline phosphatase J clin Path 20 647 653 1967

Acta path microbiol scand Section A 81, 5 651-656, 1973

GENESIS OF FOAM CELLS: STUDY IN RATS AFTER ADMINISTRATION OF INTRALIPID®

HANS FLODH and GORAN MAGNUSSON

The Toxicology Laboratories, Astra Pharmaceuticals AB, Sodertalje, Sweden

The genesis of foam cells was studied by oral administration of 20 per cent Intralipid® to rats for 14 weeks Each animal was given 1 5 ml daily The treatment induced a statistically significant increase in the number of blood monocytes and focal accumulation of foam cells in the lungs. The observations indicate that there is a relationship between the increased number of blood monocytes and the pulmonary foam cells and that blood monocytes may be transformed into foam cells in the lungs It is possible that excess lipid is picked up by monocytes in the blood and is excreted via the lungs in the form of foam cells

Lipid laden macrophages known as foam cells participate in certain disorders e g lipid storage diseases and atherosclerosis Attention has been paid to the genesis of foam cells Foam cells in atherosclerotic lesions are considered to be derived from blood monocytes to some extent (*Cookson* 1971 *Newman et al* 1971, *Tucker et al* 1971, *Wurster & Zilversmit* 1971) Findings in a model of pulmonary lipidosis indicate that there is a relationship between pulmonary foam cells and blood monocytes and that foam cells may be derived from blood monocytes (*Flodh & Magnusson* 1973)

It has been found that a diet with a high fat content results in an accumulation of foam cells in the lungs in rats (*Bernick & Patek* 1961 *Jennings et al* 1965) rabbits (*Wadell et al* 1954) and gerbils (*Bergman & van der Linden* 1971) The appearance of pulmonary foam cells is also a spontaneous disorder in the rat (*Yang et al* 1966 *Innes et al* 1967 *Giddens & Whitehair* 1969 personal observations) The aim of the present study was to investigate whether there is a relationship between the accumulation of foam cells in the lungs and an increase in the number of blood monocytes in rats given a large amount of fat orally

Received 2 ii 73 Accepted 20 ii 73

Requests for reprints should be addressed to *Goran Magnusson* The Toxicology Laboratories Astra Pharmaceuticals AB 151 85 Sodertalje

MATERIALS AND METHODS

In all 40 young male and female rats of the Sprague-Dawley strain with an initial weight of about 250 g were used as experimental subjects The animals were given food and water ad libitum The rats were divided into two groups each consisting of 10 males and 10 females The control group was not treated The rats of the experimental group were given 20 per cent Intralipid® by a daily dose of 1 5 ml per animal by oral administration for 14 weeks Intralipid® is a lipid emulsion, containing fractionated soy bean oil emulgated with fractionated egg phosphatides One litre of 20 per cent Intralipid® corresponds to 2000 kilocalories The Intralipid® was administered once a day and was always given by gavage

The animals were weighed once a week and the amount of food consumed was recorded Blood samples were taken from all control and experimental rats after 2 4 8 10 12 and 14 weeks The rats

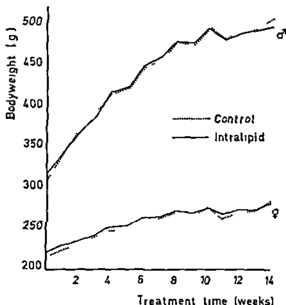


Fig 1 Weekly, mean body weights of 20 control rats and 20 rats treated daily with 1 5 ml 20 per cent Intralipid® for 14 weeks

were starved over night before blood sampling The blood samples were examined for the number of white blood cells and differential counting of leucocytes us ng standard methods At sacrifice after 14 weeks the lungs were gross examined Tissue samples of the lungs were fixed in 10 per cent neutral formalin solution for microscop c examination Paraffin embedded sections were prepared and stained with haematoxylin eosin Frozen sections were stained with haemalum and Sudan III for detection of fat

The calculated total number of white blood cells of each cell type was statistically compared in control and treated animals by means of an analysis of variance in which the three factors treatment time and animal were considered The analysis of variance was carried out on an IBM computer

RESULTS

During the whole experimental period the food cnsumption and body weight gain was the same for control and treated rats (Fig 1)

The haematological examination showed no important differences between the two groups concerning the number of leucocytes As regards the monocytes differential counting of the leucocytes revealed differences between control and treated animals (Table 1, Fig 2) In the treated rats the number of monocytes most often was 5 10 times higher than that in the controls According to an analysis of variance, the monocyte increase in the treated rats was statistically significant ($P < 0\,001$)

The mean weight of lungs in control and treated rats was comparable Macroscopically, the lungs of treated rats occasionally displayed very small greyish foci No such foci were observed in the lungs of control rats. Microscopically, a slight focal accumulation of foam cells was observed in all the treated rats and in two male and two female control rats (Fig 3) The mononuclear foam cells appeared in the alveoli and in or attached to, the alveolar walls The foamy cytoplasm of the cells was limited by a well defined membrane Staining with Sudan III showed that the cytoplasm of the foam cells contained fine fat droplets

DISCUSSSION

A focal accumulation of foam cells in the lungs was found in all rats receiving Intralipid® and in four control animals Accumulation of pulmonary foam cells with a focal distribution is a spontaneous disorder in the rat, occurring only occasionally in young animals (*Innes et al* 1967 *Giddens & Whitehair* 1969) In the present study the accumulation of foam cells had the same appearance both in the treated animals and in the control rats being much more frequent in the former than in the latter Thus the results show that an oral administration of a large amount of fat can give rise to a focal accumulation of pulmonary foam cells in the rat. This observation is in agreement with the results of other investigations (*Bernick & Patek* 1961 *Jennings et al* 1965)

Moreover the results exhibit a statistically significant increase in the number of monocytes in rats receiving Intralipid® An elevated number of monocytes is also observed

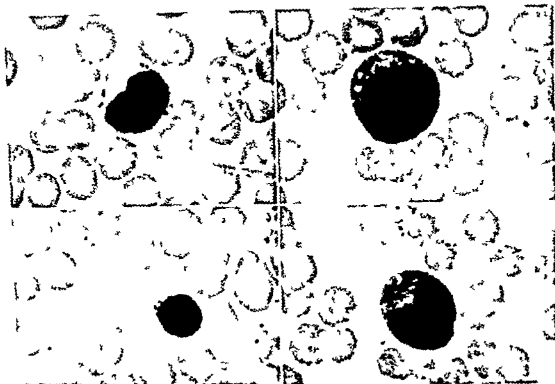


Fig 2 Three monocytes and one lymphocyte (bottom left) on a blood smear from rat after daily administration of 1 5 ml 20 per cent Intralipid® for 14 weeks The cytoplasm of the monocytes has a vacuolated appearance May Grunewald and Giemsa. × 1400

if an accumulation of pulmonary foam cells is produced by cloforex an anorogenic drug (*Flodh* & *Magnusson* 1973) Thus, experimental studies indicate that in the rat there is a monocytosis in connection with an accumulation of foam cells in the lungs It is possible that the corresponding spontaneous disorder is accompanied by an increase in the number of blood monocytes This might also be true of related conditions in which foam cells are involved

In the rat there is an excretion of lipids via the lungs (*Bernick* & *Patek* 1961 *Nicol* & *Cordingley* 1967) It is very likely that the lipid-containing foam cells are involved in this pulmonary lipid excretion Rats and rabbits receiving large amounts of dietary fat show an increase in the lipid laden monocytes which are able to transport lipids through the blood (*Simon et al* 1961 *Suzuki* & *O Neal* 1964 1967) In this investigation there was also an elevated number of monocytes, very probably containing lipid as indicated by the vacuolated appearance of the cytoplasm Thus it has been demonstrated that after administration of a large amount of dietary fat to rats, there is monocytosis and an accumulation of foam cells in the lungs and that lipid is present in the monocytes and the foam cells Furthermore it is considered that macrophages throughout the body are important for the maintenance of the normal equilibrium of serum lipids (*Day* 1967) It is possible that blood monocytes pick up excess lipid and that lipid containing monocytes on arrival in the lungs migrate from the blood vessels into the alveoli where they appear as foam cells The observations of the study indicate that the monocytes in the rat take part in the excretion of fat via the lungs

Another observation in this study may support the suggestion of an excretion of excess

TABLE 1 *Differential Counting of Leucocytes in Rats after Daily Administration of 1 5 ml 20 Per Cent Intralipid®*

Duration of experiment (weeks)	Monocytes* per cent		Lymphocytes per cent		Granulocytes							
					Neutrophilic				Eosinophilic per cent		Basophilic per cent	
					Band per cent		Seg per cent					
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
2	0 6	2 4	89 7	87 1	0 1	0 1	9 0	9 6	0 8	0 9	0 0	0 0
4	0 5	2 4	91 9	89 9	0 0	0 0	7 0	7 1	0 7	0 7	0 0	0 0
6	0 4	1 3	90 5	90 4	0 2	0 0	8 4	7 6	0 6	0 8	0 0	0 0
8	0 4	1 9	90 5	89 0	0 0	0 0	8 5	8 3	0 7	0 9	0 0	0 0
10	0 2	2 0	88 6	87 3	0 0	0 0	10 1	9 8	1 1	1 0	0 0	0 0
12	0 4	2 6	87 5	83 5	0 0	0 0	10 7	13 0	1 4	1 1	0 0	0 0
14	0 4	3 0	87 8	81 8	0 0	0 0	11 0	14 6	0 9	0 6	0 0	0 0

Each value is the *mean* of 20 single values (10 males and 10 females)
* Monocyte increase statistically significant ($P < 0\ 001$) and caused by treatment (Analysis of variance)

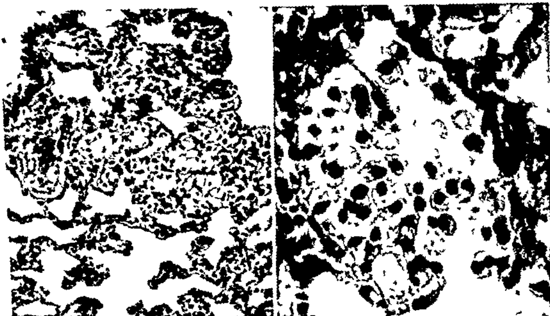


Fig 3 Foam cells in lungs of rat after daily administration of 1 5 ml 20 per cent Intralipid® for 14 weeks Haematoxylin eosin Left × 200 Right × 500

lipid The control and treated rats showed the same gain in body weight though the intake of calories of the latter had been greater due to the administration of Intralipid® If there had not been any lipid excretion there should have been a greater gain in body weight in the treated rats than in the control ones This was not the case

REFERENCES

Bergman F & van der Linden W Reaction of the mongolian gerbil to a cholesterol cholic acid-containing gallstone induced diet Acta path microbiol scand Section A 79 476–486 1971

Bernick S & Patek P R Effect of cholesterol feeding on rat reticuloendothelial system Arch Path 72 310–320 1961

Cookson F B The origin of foam cells in atherosclerosis Brit J exp Path 52 62 69 1971

Day A J Advances in lipid research Academic press New York and London 1967 p 185 207

Flodh, H & Magnusson G Genesis of foam cells Study in rats after administration of cloforex Virchows Arch Abt B 12 360–366 1973

Giddens W E & Whitehair C K Germ free biology Plenum press New York 1969 p 75–84

Innes J R M Garner F M & Stookey, J L Pathology of laboratory rats and mice Blackwell scientific publications Oxford Edinburgh 1967 p 229–257

Jennings, I W Gresham G A & Howard A N Pulmonary lipidosis in laboratory rats J Reticuloendoth Soc 2 130–140 1965

Neuman H A I Murad T M & Geer J C Foam cells of rabbit atheromatous lesion Lab Invest 25 586 595 1971

Nicol T & Cordingley J L Reticuloendothelial excretion via the bronchial tree In The reticuloendothelial system and atherosclerosis (N R D Luzio and R Paoletti eds) p 58–62 New York Plenum Press 1967

Simon R C Still W J S & O Neal R M The circulating lipophage and experimental atherosclerosis J Atheroscler Res 1 395–400 1961

Suzuki M & O Neal R M Accumulation of lipids in the leukocytes of rats fed atherogenic diets J Lipid Res 5 624–627 1964

Suzuki M & O Neal R M Circulating lipophages serum lipids and atherosclerosis in rats Arch Path 83 169–174 1967

Suzuki M Singleton M R & O Neal R M Transport of lipids by blood leukocytes Circulation 36 suppl 2 41 1967

Tucker C F Catsulis C Strong J P & Eggen D A Regression of early cholesterol induced aortic lesions in rhesus monkeys Amer J Path 65 493 514 1971

Wurster, N B & Zilversmit D B The role of phagocytosis in the development of atherosclerosis lesions in the rabbit Atherosclerosis *14* 309–322, 1971

Yang, Y H, Yang, C Y & Grice, H C Multifocal histiocytosis in the lungs of rats J Path Bact *92* 559–561, 1966

Acta path microbiol scand Section A 81, 5, 657-662, 1973

A STEREOLOGICAL STUDY OF INTRAHEPATIC BILE DUCTS

I Method and Application to Normal Livers

M Jorgensen

University Institute of Pathological Anatomy, Copenhagen, Denmark

Basic principles of stereology are discussed and their application to the study of intrahepatic bile ducts is investigated on a material of normal livers This study has demonstrated that throughout life the bile ducts in the portal tracts have a shape approximating to circular cylinders. There is probably no great variation in shape If there is any variation, however, it is unsystematic and not correlated to age or diameter of the ducts The course of the ducts is straight over quite long segments Partial parallelism is not a marked feature Mean diameter increases slightly between the newborn period and adult age The method is applicable to the distinction between relatively unrelated duct forms from their appearance in histological slides In this respect it is the only objective and quantitative method at our disposal Stereology is less suitable for the determination of ductal shape

In previous papers on abnormal bile ducts the anatomy was studied by three dimensional reconstructions (*Jørgensen* 1971 b + 1972) However, reconstructions from serial sections are very time consuming In consequence this method of investigation is applicable only to a small number of selected cases Moreover a reconstruction gives information exclusively about the reconstructed volume of tissue This information will in most cases be of no interest unless the results can be extrapolated to structures outside the reconstructed volume *believed* to have the same anatomy Our conception of identical morphology in microscopy is usually based on the subjective impression that the structures in question 'look alike in the histological slides

For practical purposes many details in a histological slide (e g bile ducts) can be considered as two-dimensional images of three dimensional objects The shapes of these images (*the profiles*) are of course not quite accidentally They are a function of the three-dimensional shape of the object and the level and direction of the section If the objects in question are sectioned in a nonpreferential manner, the distribution of profiles will be characteristic for the geometrical shape of the objects By using a geometrico-statistical evaluation of the quantitative data obtained from randomly collected profiles it will often be possible to draw conclusions about the geometrical properties of the sectioned structures The aims of the present study are

1) To give description and evaluation of the stereological method in relation to the study of intrahepatic bile ducts
2) To apply this method of investigation to normal livers

Received 24 viii 72 Accepted 13 iii 73

Request for reprints should be addressed to *M Jørgensen*, Institute of Pathology, Amtssygehuset i Gentofte 2900 Hellerup Copenhagen Denmark

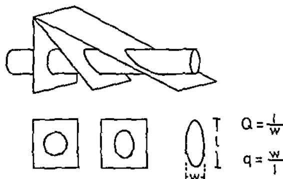


Fig 1 Different profiles obtained on section of a circular cylinder Calculation of axial ratio

METHOD AND MATERIAL

The shape of normal bile ducts would seem from reconstructions to approximate to straight tubes (*Jorgensen*, 1971 a) If the shape of these tubes were a circular cylinder (i e the locus of straight lines passing through a circle perpendicular to its plane) then the profiles from the section of such structures will be ellipses

As seen in Fig 1 the shape of the ellipses will vary with the angle of section The shape of an ellipse can be characterized by its axial ratio $Q = \frac{l}{w}$ (largest diameter over shortest diameter) If such cylinders are sectioned randomly the resultant distribution of profiles grouped according to their Q as illustrated in Fig 2 A, will be characteristic for circular cylinders This diagramatical form is less expedient because the majority of the profiles will fall into a very few of the Q classes As Q can assume values from 1 0 to ∞, the curve continues infinitely to the right. A more reasonable diagramatic approach is to classify the profiles according to the axial ratio $q = \frac{w}{l}$ The possible values of q are between 0 and 1 0 We then have only a limited number of q-classes and a more even distribution of profiles between the q classes This gives a curve approximating to a straight line In Fig 2 B this diagramatic form is illustrated with the use of accumulative percentages

An elliptical cylinder is the locus of straight lines passing through an ellipse perpendicular to its plane The q of this ellipse (the directrix) characterizes the shape of the cylinder The profiles obtained on sectioning such a cylinder will also be ellipses *Hennig* has calculated the theoretical distributions of axial ratios for random sections of different cylinder forms (*Henning* & *Elias*, 1964), as shown in Fig 3 The parabola to the right indicates the distribution for circular cylinders (q of directrix = 1 0)

The other curves apply to cylinder forms of increasing flatness from right to left, as indicated by their q of directrix

These theoretical distributions form in part the basis for the investigation of the material, which comprises liver tissue from 10 autopsies Group A consists of 5 newborn children, and group B of 5 adults, age 22, 31, 49 60 and 65 years None of the patients showed evidence of liver or biliary disease The tissue was fixed in buffered formalin embedded in paraffin and cut in sections of 5 10

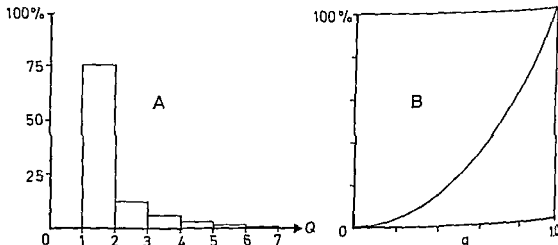


Fig 2 The percentage distribution of profiles fromrandom section of circular cylinders. The profiles are grouped according to their axial ratio (abscissa) In diagram A 'Q' is used In diagram B q is used and the figures are given in accumulative percentages

μm thickness, which were stained with hematoxylin and eosin

Only one slide from each case was used 100 profiles of sectioned bile ducts were drawn on tracing paper by means of a Leitz drawing device, so that *all* ducts in a randomly chosen 'path' were drawn Each profile was numbered and its largest and shortest diameter measured in mm. From these figures the axial ratio q was calculated

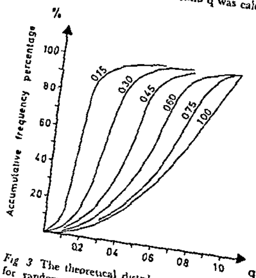


Fig 3 The theoretical distributions of axial ratios for random section of different cylinder shapes The distribution for each cylinder shape is marked with its q of directrix The diagram has kindly been provided by *Dr August Hennig*, München

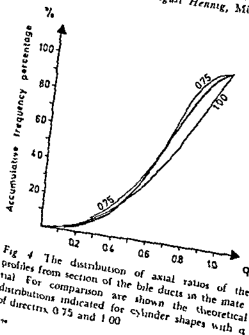


Fig 4 The distribution of axial ratios of the profiles from section of the bile ducts in the material For comparison are shown the theoretical distributions indicated for cylinder shapes with q of directrix 0 75 and 1 00

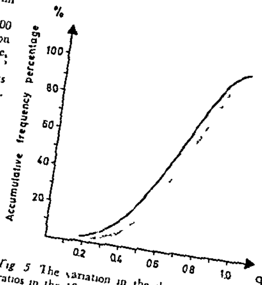


Fig 5 The variation in the distribution of axial ratios in the 10 cases of the material

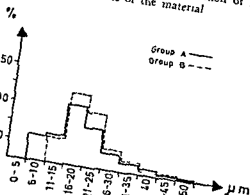


Fig 6 The percentage distribution of w in microns of the profiles in group A and group B Group A was drawn at a total magnification (microscope + drawing device) of 400 × while the profiles of group B were drawn at a total magnification of 600 ×

RESULTS

Fig 4 shows the distribution of axial ratios in the material There is no systematic difference in the distribution with respect to age in group B and no difference between groups A and B The unsystematic spread of values among the 10 cases shown in Fig 4 is illustrated in Fig 5 It has not been possible to indicate confidence limits for this small material

In Fig 6 the percentage distribution of w in microns is shown separately for groups A

TABLE 1 *The Distribution of Axial Ratios of the Profiles in Group A with Respect to w*

w μm (n)	q intervals									
	0 0 10	0 11– 0 20	0 21– 0 30	0 31– 0 40	0 41– 0 50	0 51– 0 60	0 61 0 70	0 71– 0 80	0 81– 0 90	0 91 1 00
6–10 (67)		3	4	6	15	11	9	36	0	16
11–15 (74)	1	6	4	4	12	19	13	15	19	7
16–20 (159)		3	5	8	10	13	25	19	13	4
21–25 (102)		1	1	5	15	21	23	7	25	2
26–30 (44)			3	9	2	21	27	18	18	9
31–35 (22)				13	18	10	10	18	27	4
≥ 36 (32)										

and B There is no systematical variation with respect to age in group B Mean w in group A is 20 4 μm and in group B 22 2 μm

Table 1 records the distribution of axial ratios for different values of w in group A In the group w 6–10 μm the distribution of profiles is distorted in the higher q classes, as discussed below

DISCUSSION

Stereological principles have been used for many years in astronomy, geology and mineralogy The discovery of the liver muralium (*Elias*, 1948) provided the basis for the establishment of stereology as a formal science This tool has been used in pathology only in recent years (e g studies of renal corpuscles by *Elias & Hennig, 1967*, and of human lungs by *Weibel*, 1963) To the author s knowledge it has not been applied to the study of intrahepatic bile ducts In this field the method could be useful in at least two ways

1) as basis for the assumption of identical or different geometrical forms of ductular populations
2) in the determination of ductal anatomy

A The Stereological Method

The error of drawing and measurement is significant if the profiles on the tracing paper are small, i e if the magnification used is small in proportion to the width of the sectioned ducts With respect to the distribution of axial ratios any distortion will be most pronounced in the right part of the curve (high q-classes) For the smallest infantile ducts in Table 1 the distribution of q is distorted and incorrect in the higher q-classes ... (100 ×) has been ... cation of 165 × The resulting distribution of axial ratios was different from Fig 4 and obviously distorted

The value and precision of stereology as a statistical method is dependent on the number of data available and of their non preferential collection The number of profiles from each case in this investigation—one hundred—is too small to avoid sampling error This must be responsible *in part* for the spread of values in Fig 5 The precision increases with the number of profiles drawn. The random collection of profiles can be compromized by preferential orientations of the ducts If part of the ducts runs parallel the shape of their sections will be identical This profile shape will be *encountered* too ... with respect to 'dominating duct orientations this will partly compensate for the error (e g Fig 4)

The stereological determination of the geometrical form of a structure is based on iden

tical distribution of profile shapes in the structure in question and in idealized mathematical models. Sources of error will inevitably arise from the differences between the premises drawn from these models and the actual findings. The premises for the distribution in Fig. 3 are: Straight cylinders of identical form and infinite length cut in a non-preferential way in sections of thickness zero. None of these premises can be fulfilled in any biological study. Bile ducts can only be considered as straight cylinders in short segments between bends and angulations. The finite length of the straight segments will only have a minor distorting effect on the distribution of axial ratios because very oblong profiles are rare unless the sectioned structures are really flattened (Fig. 3). The distorting effect of sections through angulations and bends must be moderate if the length of the straight segments is not too short. The problems of lack of randomisation in the selection of profiles have been discussed. The influence of section thickness is dependent on the dimensions of the cut structures. In general it must be assumed to be of no importance in this study. The premise of identical shape of the sectioned cylinders is troublesome. If the ductal population is a mixture of cylinder shapes *very* different from one another, then the resulting distribution of axial ratios will be very different from any of the theoretical distributions. In this case the only possible explanation lies in mixed ductular population. But if the cylinder shapes are more closely related, the resulting distribution of q will be close to the distribution of a cylinder shape intermediate to those of the ductal mixture. The deviation from such a theoretical distribution will only be small and indistinguishable from that which *could* be caused by the other sources of error. In this case the interpretation of ductal shape will be wrong. Only where there is a correlation between ductal shape and width *may* we have a chance to escape the wrong stereological interpretation.

Conclusion. The distribution of the two parameters q and w represents a quantification of the way in which bile ducts are seen in histological slides. On the assumption of random sampling of the profiles it is an objective characterization of ductular populations. This makes it a reasonable instrument for the distinction between populations of different morphology. The sensitivity of the method makes it unsuitable for the distinction between closely related morphologies. As a method for determination of geometrical form it is beset with several sources of error, the exact influence of which are unknown. It may give wrong results. At best it can be used to give a rough estimation of ductular form.

B. Intrahepatic bile ducts in normal livers

The shape of the graph in Fig. 4 is characteristic for straight cylinders. They must of course be of finite length, and q less than 0.10 was not encountered. Although the lengths of the straight segments cannot be determined more accurately from this study, the left part of the diagram indicates that they cannot be very short compared to their width. As to the shape of the cylinders, the frequency of profiles in the q intervals 0.1–0.4 corresponds to that of the two cylinder forms indicated. The low frequency of profiles in the q interval 0.9–1.0 is characteristic for flattened cylinders. Nevertheless it is impossible to say whether the distribution found represents a distortion of one or the other of the theoretical distributions. The possibility of mixed ductular shapes must be kept in mind (in the sense of a major systematic difference in shape over and above nature's lack of mathematical accuracy, affecting a significant proportion of the ducts). In circular cylinders w of the profiles will always be identical with the diameter of the sectioned cylinder. If the profiles are grouped according to w (= cylinder diameter) the distribution of q will still be that of circular cylinders in each group. Where elliptical cylinders are concerned both w and l will vary with the angle of section and w will not be the same as the short diameter of the directrix. But it holds true of all cylinder shapes that the majority

of profiles will be grouped around the form of the directrix

In Table 1 the profiles are grouped as outlined above The number of profiles in each group (n) is so small that it is rather dangerous to draw conclusions from these figures But the result does not support the assumption that the size ("diameter") of the ducts is correlated to any major difference in duct shape An unsystematic variation in shape cannot, of course, be excluded, but is perhaps less probable If the shape of the ducts is relatively uniform then it must approximate to a circular cylinder In this case the distribution of w in Fig 6 must be close to the distribution of duct diameters The augmentation of mean duct diameter from the new born period to the adult state could be achieved in several ways One possibility is general growth of all the ducts while a growth or disappearance of the smallest infantile ducts is another

The diagram in Fig 6 could be interpreted in favour of the latter possibility But the material is too small to draw any definite conclusions in this respect Partial parallelism of the ducts may influence the distribution of axial ratios as already discussed From Fig 5 it is evident that this probable partial parallelism cannot possibly be as pronounced as is generally believed

Conclusion The bile ducts in normal livers are not tortuous structures Throughout life they have a straight course over quite long segments. There may be some minor degree of partial parallelism between the ducts but this is not a dominant feature The shape of the ducts approximates to a circular cylinder It is not probable that there is any greater variation in shape, especially correlated to age or diameter There is a moderate increase in mean duct diameter from the new born period to adult life In adults the anatomy seems constant

---

The author wishes to thank *Dr Jakob Visfeldt* Copenhagen, and *Dr August Hennig*, Actuary München, for discussions and help This work was supported by a grant from *Kong Christian den Tiendes Fond*'

REFERENCES

Elias, H Do liver cords exists? Anat Rec *100* 23–23, 1948

Elias, H & *Hennig, A* Stereology of human glomerulus In Quantitative methods in morphology Ed *Weibel E R* & *Elias H* Springer Verlag Berlin Heidelberg New York 1967

Hennig, A & *Elias, H* Elliptical cylinders Z wiss Mikrosk *66* 226 234 1964

Jorgensen M Three dimensional reconstruction in histology Acta path microbiol scand Sect A *79* 298 302 1971 (a

Jorgensen M A case of abnormal intrahepatic bile duct arrangement submitted to three-dimensional

scand

Jorgensen

intrahepatic bile ducts in a case of polycystic disease of the liver in an infant Acta path microbiol scand Sect A *80* 201 206 1972

Weibel E R Morphometry of the human lung Springer Verlag Berlin Heidelberg New York 1963

Acta path microbiol scand Section A 81, 5, 663-669, 1973

A STEREOLOGICAL STUDY OF INTRAHEPATIC BILE DUCTS

2 Bile Duct Proliferation in Some Pathological Conditions

M Jorgensen

University Institute of Pathological Anatomy Copenhagen, Denmark

An anatomical investigation of 'bile duct proliferation' has been undertaken in order to contribute to our understanding of its pathogenesis Stereology has not revealed any differences in anatomy which could be correlated to aetiology The geometrical shape of the ductules was found to be a flattened cylinder This explains the impression that they are sectioned more longitudinally in histological slides than normal ducts. A three dimensional reconstruction from one of the cases confirmed this result and illustrated the arrangement of the ducts These formed an irregular, three dimensional network with many connections to the parenchyma It is concluded that this anatomy differs so markedly from that of normal bile ducts that it is improbable that the latter are the source of origin In contrast, the anatomy is quite similar to that of the hepatic muralium which is proposed as the origin of proliferating ductules

In liver pathology 'bile duct proliferation" is one of the most frequent changes of the biliary system It is encountered in many liver diseases of completely different aetiology This form of reaction is only poorly understood with respect to its pathogenesis and functional implications Part of the reason for this is lack of adequate information about the anatomy of these structures Previous studies of 'proliferating ductules" have primarily been concerned with the question of continuity of the ductal system If the system were at some level (e g the ductular) discontinuous this might offer a mechanical explanation for intrahepatic bile stasis For this purpose some reconstructions have been made as discussed below But apart from its capacity to drain bile, the ductal anatomy has attracted very little interest

"Proliferating ductules" have to some extent a different appearance from normal ducts in histological slides This is reflected in the terms atypical ductular or pseudoductular proliferation and cholangiolaminae Insofar as this difference in appearance concerns the shape of the sections (the profiles), it must reflect a difference in the geometrical shape or the course of the ducts

The present investigation is carried out by means of stereology and three-dimensional reconstructions As previously discussed, both methods have limitations and drawbacks (*Jorgensen* 1973) A combination of the two methods is rational as they are to some extent supplementary

The aim of this study has been to use an anatomical investigation to contribute to the discussion about the pathogenesis of 'bile duct proliferation'

Received 23 ii 73 Accepted 23 iv 73

Requests for reprints should be addressed to M Jorgensen Institute of Pathology Amtssygehuset i Gentofte 2900 Hellerup Copenhagen Denmark

TABLE 1. *Data of the 5 Patients*

Case no	Sex	Age	
1	M	48	Unmarried waiter For many years heavy beer-drinker Autopsy diagnoses cirrhosis of the liver, duodenal ulcer and chronic pancreatitis
2	F	52	Cirrhosis of the liver was an incidental autopsy finding The clinical report did not contain any information about previous liver diseases Main diagnoses severe atherosclerosis and myocardial infarction
3	F	9/12	At laparotomy at 2 months of age the diagnosis was atresia of extrahepatic bile ducts Six months later attempt at surgical drainage operation
4	F	89	Gall stones for many years On admission occlusive jaundice Autopsy revealed biliary cirrhosis, gall stones in all extrahepatic bile passages and a recent thrombosis of portal vein adjacent to a large gall stone in the common duct
5	M	14	Secondary haemochromatosis Aplastic anaemia treated with multiple transfusions Thyreoid deficiency, diabetes mellitus cardiac enlargement and insufficiency

MATERIAL

The material consists of liver tissue from 5 patients with different liver diseases Detailed data are given in Table 1 Common to all 5 cases was the fact that "bile duct proliferation" was a very conspicuous feature of the histological picture (Figs 1 2 and 3) This tissue was fixed in buffered formalin and embedded in paraffin For the stereological study sections of about 10 μm thickness were cut and stained with hematoxylin and eosin From case no 3 serial sections of 5 μm thickness were cut for the reconstruction, the same staining was employed

METHODS

The stereological method has previously been described in detail (*Jorgensen* 1973) Briefly, one hundred sectioned bile ducts chosen at random from each case are drawn on tracing paper Total magnification (microscope + drawing device) is 600 × The long diameter (l) and the short diameter (w) of these profiles are measured. The axial ratio $q = \frac{w}{l}$ is calculated for each profile The profiles are grouped according to their shape (q) The resulting percentage distribution of axial ratios is compared with the theoretical distributions of different cylinder shapes

The technique of three-dimensional reconstruction used has also been described in detail earlier (*Jorgensen* 1971) Briefly the technique involves the transfer of drawings of serial sections to acryl plates After being glued together the plates constitutes a highly transparent block The total magnification was 200 ×, and the thickness of the acryl plates 1 mm

RESULTS

The distribution of axial ratios for the total material is showed in Fig 4 The three theo-



Fig 1 Case no 2 Cirrhosis of unknown aetiology H E 40 ×

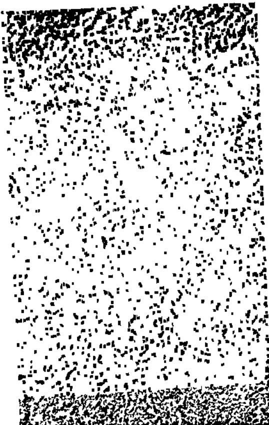


Fig 2 Case no 4 Biliary cirrhosis with abundant "proliferating bile ductules" H E 40 ×

retical distributions indicated in the diagram are those of straight cylinders with axial ratios of directrix 1 00–0 75 and 0 60 (Directrix = the profile of an exact transverse section of the cylinder)

The variation in the distribution of axial ratios among the 5 cases is illustrated in Fig 5 The material is too small to indicate confidence limits

Fig 6 gives the percentage distribution of w in microns

In Fig 7 the result of the reconstruction is shown The "proliferating ductules" are not small, short and blindly-ending tubes connected with the parenchyme Nor, in this reconstruction, do they seem [illegible]

[illegible] to the parenchyme A most interesting feature of these "proliferating ductules" has been difficult to illustrate in the photographs As a consequence of the technique employed for the reconstruction, nothing can be seen when the block is viewed directly from the side It has to be tilted and viewed at an oblique angle with respect to the sides, or else to be inspected through the top or bottom. But by comparing Fig 7 d and Fig 7 h it can be seen that the ductules are *flattened.* A closer inspection of the reconstructed block reveals that the direction of flatness does not have any preferential orientation, but varies as we follow the ducts in the network A measurement of the directrix of the ductules at different points of the network has not been possible on the block An *estimation* from inspection is q about 0 5 Evidently there is some variation in the

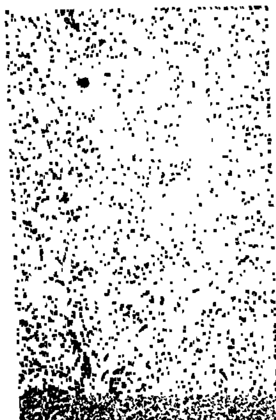


Fig 3 Case no 5 Secondary haemochromatosis The liver was loaded with iron H E 40 ×

TABLE 1 *Data of the 5 Patients*

Case no	Sex	Age	
1	M	48	Unmarried waiter For many years heavy beer drinker Autopsy diagnoses cirrhosis of the liver duodenal ulcer and chronic pancreatitis.
2	F	52	Cirrhosis of the liver was an incidental autopsy finding The clinical report did not contain any information about previous liver diseases Main diagnoses severe atherosclerosis and myo cardial infarction
3	F	9/12	At laparotomy at 2 months of age the diagnosis was atresia of extrahepatic bile ducts Six months later attempt at surgi cal drainage operation
4	F	89	Gall stones for many years On admission occlusive jaundice Autopsy revealed biliary cir rhosis gall stones in all extra hepatic bile passages and a re cent thrombosis of portal vein adjacent to a large gall stone in the common duct
5	M	14	Secondary haemochromatosis Aplastic anaemia treated with multiple transfusions Thyreoid deficiency diabetes mellitus cardiac enlargement and in sufficiency

MATERIAL

The material consists of liver tissue from 5 patients with different liver diseases Detailed data are given in Table 1 Common to all 5 cases was the fact that 'bile duct proliferation was a very conspi cuous feature of the histological picture (Figs 1 2 and 3) This tissue was fixed in buffered formalin and embedded in paraffin For the stereological study sections of about 10 µm thickness were cut and stained with hematoxylin and eosin From case no 3 serial sections of 5 µm thickness were cut for the reconstruction the same staining was employed

METHODS

The stereological method has previously been de scribed in detail (*Jorgensen* 1973) Briefly, one hundred sectioned bile ducts chosen at random from each case are drawn on *tracing paper* Total magnification (microscope + drawing device) is 600 × The long diameter (l) and the short diameter (w) of these profiles are measured. The axial ratio $q = \frac{w}{l}$ is calculated for each profile. The profiles are grouped according to their shape (q) The resulting percentage distribution of axial ratios is compared with the theoretical distributions of different cylinder shapes

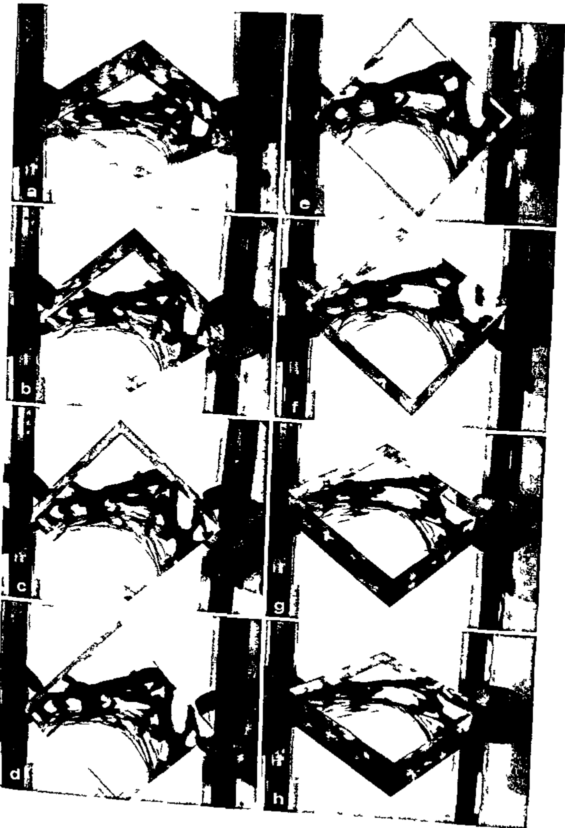
The technique of three dimensional reconstruc tion used has also been described in detail earlier (*Jorgensen* 1971) Briefly the technique involves the transfer of drawings of serial sections to acryl plates After being glued together the plates constitutes a highly transparent block The total magnification was 200 ×, and the thickness of the acryl plates 1 mm

RESULTS

The distribution of axial ratios for the total material is showed in Fig 4 The three theo-



Fig 1 Case no 2 Cirrhosis of unknown aetiology H E 40 ×


a
b
c
d
e
f
g
h

zation of ductular populations, on the assumption of random sampling of the profiles. The sensitivity of the method was not sufficient for it to be suitable for the distinction between different but closely related morphologies.

In Fig. 5 the spread in the distribution of axial ratios among the five individual cases is illustrated. This spread is not greater than was the case in normal bile ducts (*Jorgensen* 1973). It is probably to a great extent caused by sampling error secondary to the small number of profiles (one hundred) from each case. We have no reason to believe that the premise of randomization should be less completely fulfilled than was the case for normal ducts. The reconstruction in Fig. 7 does not provide support for the assumption that there is any great degree of parallelism among the straight segments of proliferating ductules. The variation in the distribution of w is also small, as illustrated in Fig. 6. We can conclude that, within the sensitivity of the stereological method as applied here, no difference has been demonstrated among the ductal populations of the five cases. It is highly probable that *"bile duct proliferation" is a morphological unity irrespective of aetiology*.

The interpretation of axial ratio distribution in terms of ductular shape is beset with several sources of error, as discussed previously (*Jorgensen* 1973). The graph in Fig. 4 is not identical with any of the theoretical distributions indicated. But the shape of the graph is characteristic for straight cylinders. They must of course be of limited length and $q < 0.10$ was not encountered. But from the left part of the graph it may be concluded that the length of these straight cylinder segments cannot be very short compared to their width. The distribution found could be the result of "mixed ductular population", i.e. ducts of different geometrical shape. This was to be expected, as a part of the sectioned ducts are probably normal and thereby approximate in shape to circular cylinders (*Jorgensen* 1973). At all events this applies to the less advanced cases of the liver diseases dealt with here. Apart from the mixture of normal and abnormal ducts, the reconstruction demonstrated some variation in shape among the abnormal ducts. The exact shape of the proliferating ductules or the composition of the mixture of ductal shapes cannot be seen from the graph in Fig. 4. But the frequency of profiles in the left half of the diagram (q 0.1–0.5) cannot be obtained unless a substantial part of the ducts have a q of directrix of 0.60 or less. It may therefore be concluded that *proliferating ductules are flattened cylinders* and thereby different in shape from normal ducts.

In elliptical cylinders w of the profile is not identical with the short diameter of the directrix (*Jorgensen* 1973). But the distribution of w in Fig. 6 gives some information about how the width of the sectioned ducts appears in the histological slides. If the distribution in Fig. 6, Diagram B is compared with the similar distribution of normal ducts in adults (*Jorgensen* 1973), it is seen that the distributions are practically identical. The statement that proliferating ductules are particular small compared with normal ducts is consequently incorrect.

There is some variation in the distribution of w among the five cases (Fig. 6). This is probably accidental and caused by sampling error. The possibility of a real difference can of course not be excluded. Real differences in distribution of w *do* exist. In cases of cirrhosis of the liver adjoining areas sometimes show proliferating ductules of different width. This could be interpreted as areas in which the proliferating ductules were dilated secondary to collapse of tissue causing compression of the efferent biliary pathways from the area in question. But such a simple mechanical explanation is not necessarily true. In case no. 2 the phenomenon was present. One hundred profiles were drawn and measured from an area with large proliferating ductules (mean w 27 μm) and compared with one hundred profiles from an area with "small proliferating ductules" (mean w 24 μm). The distribution of axial ratios was exactly the same, indicating that the morphology was the same. If simple

hydrodynamic factors were responsible for the larger width, one would expect the shape of these "dilated ducts" to approximate to that of a circular cylinder It should be emphasized that the above applies to bile ducts without visible bile stasis

The two main features of the reconstruction in Fig 7 are the three dimensional network of the proliferating ductules and their flattened shape The network or labyrinth architecture cannot be seen from a histological slide, nor is it revealed by the stereological study It has previously been demonstrated by reconstructions (*Takahashi* & *Hayama* 1967, *Ryoji Oh i et al* 1969) This architecture is in striking contrast to the normal pattern of bile ducts, characterized by an approximately straight course and practically no interconnections (*Takahashi* & *Hayama* 1967, *Ryoji Oh-i et al* 1969)

To the author's knowledge the flattened shape of the ductules has not been described previously As the reconstructions refered to above are of the graphic type, it is impossible to judge ductal shape from the illustrations The flattened shape was to be expected, as it is the only reasonable explanation of the fact that proliferating ductules are sectioned longitudinally more commonly than normal ducts As discussed, this shape cannot primarily be determined by hydrodynamic factors A possible explanation is that normal function has not sculptured these structures into their usual shape If this is true then the geometrical shape tells more about origin and pathogenesis than about function

In cirrhosis the biliary system is changed on almost every level *Masuko et al* (1964) have described the changes in the larger ducts from neoprene corrosion casts These changes can only be explained by proliferation of ductal epithelium With respect to the order of ducts dealt with in this study, no agreement has been reached as regards their derivation The two most probable sources of these ductules are the preexisting ductal system and the hepatic parenchyma Circumstantial evidence has been presented in favour of both possibilities The result of the present investigation supports a parenchymal origin. Both the network arrangement of the ducts and their flattened shape are difficult to explain as a result of a proliferation of normal bile ducts, in consideration of their shape and course The anatomy is much more in accordance with that of the parenchyma the muralium During the embryological development the ducts arise from the muralium by a transformation or differentiation of the hepatocyte in contact with the mesenchyme (*Elias* & *Sherrick* 1969) Such a transformation of parenchymal cells could perhaps also take place in later life

REFERENCES

1 *Elias, H* & *Sherrick, J C* Morphology of the liver Academic Press New York and London 1969 p 233–240

2 *Jorgensen, M* Three dimensional reconstruction in histology Acta path microbiol scand Sect A *79* 298–302, 1971

3 *Jorgensen M* A stereological study of intra hepatic bile ducts 1 Method and application to normal livers Acta path microbiol scand Sect A *81* 657–662, 1973

4 *Masuko, K, Rubin E* & *Popper, H* Proliferation of bile ducts in cirrhosis Arch Path (Chicago) *78* 421–431 1964

5 *Oh i, R Kasai, M* & *Takahashi T* Intra hepatic biliary obstruction in congenital bile duct atresia Tohoku J exp Med *99* 129–149, 1969

6 *Takahashi, T* & *Hayama T* Pathogenesis of so-called intrahepatic cholestasis based on three dimension analysis of the cholangiolar system Tohoku J exp Med *91* 149–176 1967

Acta path microbiol scand Section A *81, 5*, 670–675, 1973

A STEREOLOGICAL STUDY OF INTRAHEPATIC BILE DUCTS

3 Infantile Polycystic Disease

M JORGENSEN

University Institute of Pathological Anatomy, Copenhagen, Denmark

10 cases of "infantile polycystic disease" have been studied by stereology in order to investigate the general validity of the conception of the intrahepatic biliary system obtained from two reconstructions The results confirm those of the reconstructions The ductal structures can be divided into two separate groups of very different morphology One consists of very flat elliptical cylinders called ductal plates and the other of irregular tubular structures approximating to the shape of circular cylinders The two components show no preferential widths The structures are dilated compared to normal ducts, but in a very variable manner, and cysts are very rare It is concluded that the mixture of different ductal shapes constitutes a characteristic entity If it is considered desirable to use a morphological designation of this liver lesion then this should refer to this entity or to its most characteristic element, the "ductal plate"

The background for this series of bile duct studies in different liver diseases was interest in the histological picture of the liver in a case of infantile polycystic disease The bile ducts in the portal tracts were—compared to normal ducts—surprisingly often sectioned longitudinally This picture was intuitively interpreted three dimensionally as resulting *from the section of plate-formed ductal structures* The correctness of this interpretation was proved by reconstructions (*Jørgensen* 1971 & 1972) and the "ductal plate" was considered to originate from a failure in the earliest stages of bile duct embryology

As discussed in the first paper in this series (*Jørgensen* 1973 a) stereology provides an instrument for the objective characterisation of ductal populations As no such general characterisation of the bile duct structures in infantile polycystic disease has yet been made, a material of 10 cases has been investigated in this way In combination with the detailed information obtained from the reconstructions, this gives a total picture of the biliary system in this disease

The nomenclature is discussed on the basis of these findings

MATERIAL

The material consists of liver tissue from 10 newborn infants, all stillborn or dying shortly after the birth

In all the cases both kidneys were considerably enlarged and transformed into cystic and sponge like structures Fig 1 shows the typical oblong and radially appearance of the kidney cysts.

All the livers were grossly normal except one (case 1) which displayed a multitude of cysts On microscopy of liver tissue the characteristic picture of the bile ducts was found in all the cases (Fig 2)

Received 13 iii 73 Accepted 13 iii 73

Request for reprints should be addressed to *M Jørgensen*, Institute of Pathology, Amtssygehuset i Gentofte, 2900 Hellerup, Copenhagen, Denmark

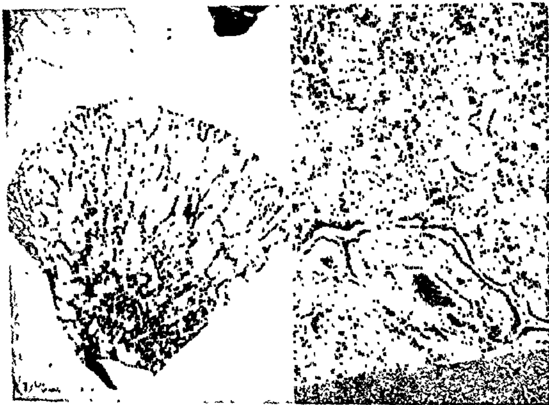


Fig 1 Th　, .al appearance of the kidneys in 'infantile polycystic disease' From case 9 Magnification 5 ×

Fig 2 The characteristic appearance of the biliary structures in the portal tracts From case 9 Van Gieson stain 40 ×

Seven of the children had no malformations apart from those of the kidneys and the liver, while three had additional multiple and severe malformations of other organ systems

Cases 1 and 5 have been reported previously (*Jørgensen* 1971 & 1972)

METHOD

The stereological method applied has previously been described in detail (*Jørgensen* 1973 a) Briefly 100 sectioned bile ducts chosen at random from each case were drawn on tracing paper For each of the numbered profiles the length (l) and the width (w) were measured in mm Where the lumen branched the l of the individual branches were added Where there were variations in the width of the profile an average was used for w

For each profile the axial ratio $q = \frac{w}{l}$ was calculated The percentage distribution of profile shapes (q) is compared with the distribution of known geometric shapes

RESULTS

The distribution of axial ratios from the sectioned ducts in the material is given in Table 1 In order to investigate the influence of magnification, half of the material was also examined under a higher magnification The resulting distribution of axial ratios is given in Table 2 The results are illustrated diagrammatically in Fig 3 which also shows the theoretical distributions of axial ratios for random sections of straight cylinders with q of directrix 0 15 and 0 60 (directrix = the profile from an exact transverse section of a straight cylinder)

TABLE 1 *Percentage Distribution of Axial Ratios (q) of the Sectioned Ducts Total Magnification 165 ×*

→ q	0.00–0.10	0.10–0.20	0.20–0.30	0.30–0.40	0.40–0.50	0.50–0.60	0.60–0.70	0.70–0.80	0.80–0.90	0.90–1.00
Case 1	6	15	16	6	17	5	15	8	9	3
Case 2	14	24	23	10	14	2	4	7	1	1
Case 3	7	19	17	14	15	11	11	3	1	2
Case 4	16	28	18	14	7	1	8	5	0	3
Case 5	8	22	20	14	13	6	6	5	3	3
Case 6	7	21	24	14	15	7	4	6	2	0
Case 7	5	32	14	13	19	6	5	2	3	1
Case 8	9	22	15	15	11	9	9	8	0	2
Case 9	18	19	11	13	7	10	4	6	4	8
Case 10	13	14	12	15	13	12	8	5	6	2
Average	10.3	21.6	17.0	12.8	13.1	6.9	7.4	5.5	2.9	2.5

TABLE 2 *Percentage Distribution of Axial Ratios (q) of the Sectioned Ducts Total Magnification 600 ×*

→ q	0.00–0.10	0.10–0.20	0.20–0.30	0.30–0.40	0.40–0.50	0.50–0.60	0.60–0.70	0.70–0.80	0.80–0.90	0.90–1.00
Case 1	13	22	12	11	12	9	8	7	6	0
Case 2	12	27	21	14	14	3	6	1	2	0
Case 3	11	24	14	11	12	12	8	4	4	0
Case 4	16	17	12	9	13	12	13	3	3	2
Case 5	14	13	13	10	16	15	6	6	4	3
Average	13.2	20.6	14.4	11.0	13.4	10.2	8.2	4.2	3.8	1.0

Fig 4 shows the spread in axial ratio distribution among the 10 cases It has not been possible to indicate confidence limits from this study

Fig 5 illustrates the percentage distribution of w in microns Mean w in the 10 cases ranged from 25 μm to 56 μm The distributions of the extremes are shown The patient with macroscopic cysts in the liver (case 1) had a mean w and a distribution of w very close to the average for the material (The figures for case 1 refer to the ductal structures *apart* from the macroscopic cysts)

DISCUSSION

The spread in distribution of axial ratios among the 10 cases, illustrated in Fig 4, is quite pronounced compared to that of normal bile ducts (*Jorgensen* 1973 a) and bile duct proliferation (*Jorgensen* 1973 b) This raises the question of whether we are dealing with a morphological entity or not As discussed in the first paper in this series the

But if the ductal populations are composed of mixtures of very different cylinder shapes, the effect of sampling error would be expected to be pronounced This proved to be the case in the 5 cases studied in duplo In every one of the cases the difference in distribution of q between the two examinations was pronounced but the average distributions were very similar (Tables 1 and 2) The profiles

bution of ductal population, it is reasonable to conclude that *we are dealing with a ductal morphological entity*

The interpretation of axial ratio distribution in terms of ductal shape is beset with several sources of error, as previously discussed (*Jørgensen* 1973 a)

The high frequency of profiles in the q-interval 0 00 til 0 10 in the diagram Fig 3 can only be explained if a substantial part of the sectioned structures have an axial ratio of directrix of less than 0 15 The high frequency of profiles in the highest q-classes presupposes that a substantial part of the sectioned structures have a q of directrix higher than 0 60 In this way the structures can be divided into at least two major groups of

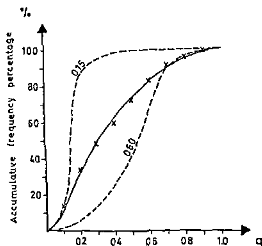


Fig 3 The percentage distribution of axial ratios (q) of the sectioned ducts
——— total magnification 165 ×
xxxxxxxx total magnification 600 ×
For comparison the distributions of axial ratios for randomly sectioned elliptical cylinders with q of directrix 0 15 and 0 60 are indicated

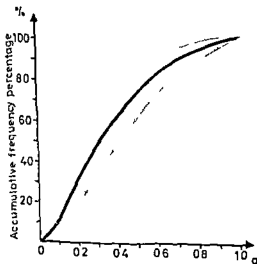


Fig 4 The spread in distribution of axial ratios among the 10 cases

selected from each case have not been the same in the two investigations and the number of profiles—one hundred—has not been large enough to avoid sampling error Taking into consideration this point, and the fact that the distributions in Fig 3 & Fig 5 are so very different from any other known distri-

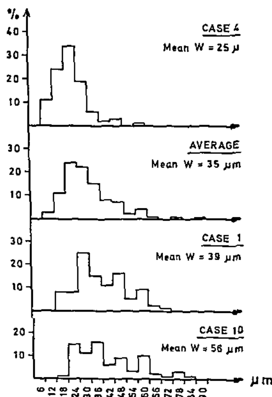


Fig 5 The percentage distribution of w in microns In addition to the average distribution for the total material the figures of three selected cases are illustrated In case 10 22 per cent of the measurements were higher than 84 μm, these are not shown in the diagram

evidently different morphology All attempts to divide the profiles into two of the theoretical distributions for different cylinder shapes (*Jorgensen* 1973 a) have been unsuccessful The reason could be that the two "extreme" morphologies are bridged by transitional geometrical shapes, or that at least one of the "extreme" shapes is heterogeneous i e comprises several related but different shapes

Another possible means of stereological separation of the different ductal shapes is based on the assumption that they may have preferential widths

It is conceivable that the ducts with a characteristic (the *q* of directrix) about 0 10 systematically had a higher *w* of their profiles than the ducts with a characteristic about 0 50, or vice versa The profiles were therefore grouped according to *w* But the distribution of axial ratios approximated in all the groups to the average of the total material We can therefore conclude that *the different ductal morphologies do not have preferential widths*

This explains the "missing" distorsion in Fig 3 secondary to the use of the low magnification 165 × Although a substantial part of the profiles are of low *w* (Fig 5), the average distribution also applies to them Consequently the combination of small *w* and high *q*-class is rare (in contrast to normal ducts) and the error arising from the use of the small magnification insignificant with respect to axial ratio distribution

If the distribution of the two stereological parameters *q* and *w* are compared with the similar distributions for normal bile ducts (*Jorgensen* 1973 a) it is evident that normal bile ducts can only constitute a *very small* part of the ducts in these patients This possibility of a small admixture of normal ducts in the pathological structures is of course not proof of their existence

The composition of the ductal mixture cannot be determined stereologically It can be done by means of reconstructions Cases no 1 and 5 have been submitted to three dimensional reconstruction as mentioned above As was to be expected from this stereological study, the reconstructions gave in principle identical results Briefly, the ductal structures consist of two distinct morphological types without transitional forms One corresponds to a very flat elliptical cylinder, often bent with respect to its long axis so as partially to encompass a vessel or a core of dense fibrous tissue (see Fig 2). These structures, called *the ductal plates*, are not of uniform dimensions The other component of the mixture corresponds to tubes approximating to the shape of a circular cylinder but irregular in form and with a very irregular course The results of the two forms of investigation are in accordance and supplementary

A patho-anatomical designation of the liver lesion should refer to the characteristic feature of the anatomy Apart from Kongenitaler Leberzirrhose , which focuses on the parenchyma (which is not cirrhotic) and 'congenital hepatic fibrosis', focusing on the portal tract fibrosis, most designations correctly call attention to the bile ducts Examples are "fibroangioadenomatose biliaire" and "polycystic disease of the liver' But both these names arise from misinterpretation of the flat, two dimensional histological picture The ducts are dilated compared to normal ducts, but in a very variable manner, and cyst formation is the exception both grossly and on the microscopical level The characteristic feature is not this variable dilatation but the mixture of different ductal shapes, of which the 'ductal plate is the most remarkable component In consequence the designation *the "ductal plate malformation"* is proposed for this hepatic lesion

The malformation is not specific for the clinical entity dealt with here It is also the liver lesion corresponding to the clinical entity congenital hepatic fibrosis", characterized by portal hypertension in children and young adults (*Kerr et al* 1961)

The malformation is never encountered in the adult form of polycystic disease of the kidneys In this disease hepatic involvement is not invariable, and when present is of quite different morphology

This work was supported by a grant from Kong *Christian den Tiendes Fond*

REFERENCES

Jorgensen, M A case of abnormal intrahepatic bile duct arrangement submitted to three dimensional reconstruction. Acta path microbiol scand Sect A. *79* 303–306, 1971

Jorgensen, M Three dimensional reconstruction of intrahepatic bile ducts in a case of polycystic disease of the liver in an infant Acta path microbiol scand Sect. A *80* 201–206, 1972

Jorgensen, M A stereological study of intrahepatic bile ducts 1 Method and application to normal livers Acta path microbiol scand Sect A *81* 657–662, 1973

Jorgensen, M A stereological study of intrahepatic bile ducts 2 Bile duct proliferation in some pathological conditions Acta path. microbiol scand Sect A *81* 663–669, 1973

Kerr, D N S, Harrison, C V, Sherlock, S & Walker, R M Congenital hepatic fibrosis Quarterly J Med *30* 91–117, 1961

Acta path microbiol scand Section A. *81, 5*, 676-680, 1973

HETEROTOPIC GASTRIC EPITHELIUM IN THE DUODENUM AND ITS CORRELATION TO GASTRIC DISEASE AND ACID LEVEL

AA JOHANSEN and O HART HANSEN

The Gastroenterological Section, Department of Pathology and the Department of Gastroenterological Surgery A, Bispebjerg Hospital, Copenhagen, Denmark

The frequency of gastric mucosal heterotopia with parietal cells in the duodenal cuff of 250 gastric resection specimens and in 22 duodenal resection specimens was studied 22.4 per cent of the gastric specimens disclosed parietal cell heterotopia The frequency was largest in specimens with prepyloric or duodenal ulcers, and a statistically significant variation with the underlying disease was found The gastric acid output was estimated in 203 cases There was a statistically significant correlation between prevalence of the heterotopia and the acid output This correlation was also evident in the individual gastric diseases In the duodenal resection specimens, gastric heterotopia was found in 22.7 per cent The heterotopia was localized in the first part of the duodenum in all cases, in one case it was also found in the second part The significance of the phenomenon for the endoscopist and the pathologist is mentioned

Heterotopic gastric mucosa in the duodenum has previously been considered as an occasional finding of no importance It presents itself either as fully mucosa heterotopia where parietal cells and sometimes peptinogen cells are seen together with gastric surface epithelium Sometimes the heterotopia is reduced to surface epithelium cells Only a few reports have dealt with the subject (*Taylor* 1927, *James* 1964, and *Hoedemaeker* 1970) Hoedemaeker observed the heterotopia to be significantly more common in specimens with duodenal ulcers than in other gastric diseases

The extensive use of duodenoscopy has increased the need for exact knowledge on the histological variation of this area This paper will describe the occurrence of gastric elements in the duodenal cuff of 250 gastric resection specimens and relate their frequency to the type of gastric diseases and to the acid secretion Futhermore, the occurrence of the heterotopia in twentytwo duodenal resection specimens will be described

MATERIALS AND METHODS

250 gastric resection specimens with a duodenal border measuring at least 15 mm and histologically examined in at least three different sections were collected consecutively from a large material All were stained with haematoxylin and eosin and with the *Marks & Drysdale* (1957) modification of the Zimmermann's staining reaction

The duodenal resection specimens were obtained from operations for pancreatic or papilla of Vater carcinoma. Several sections were taken from all parts of the specimen In the following only hetero-

Received 5.iii.73 Accepted 5.iii.73

Requests for reprints should be addressed to *Aa Johansen*, Department of Pathology, Bispebjerg Hospital, DK-2400 Copenhagen

topic areas containing parietal cells will be considered The heterotopia was not analyzed quantitatively but was recorded as present, even if only a few parietal cells were demonstrable in only one of the three sections. The acid output had been estimated using the augmented histamine test

RESULTS

The histological identification of the heterotopia was easy The parietal cells appeared bright yellow, pepsinogen cells were brownish The strong PAS positive gastric surface epithelium contrasted against the duodenal epithelium, where only the goblet cells and the brush border were stained A representative island of parietal cells is seen in Fig 1



Fig 1 A typical island of heterotopic parietal cells in the duodenum A single foveola with gastric surface epithelium is also demonstrated PAS x 100

TABLE 1 *Prevalence of Parietal Cell Heterotopia According to Sex*

Sex	No of cases	No of cases with heterotopia
Male	156	34 (21 8%)
Female	94	22 (23 4%)
Total	250	56 (22 4%)
p>0 05		

Pepsinogen cells were often, but not constantly found together with the parietal cells In none of the specimens were parietal cells demonstrated without gastric surface epithelium All heterotopic elements were situated above the muscularis mucosae

Among the 250 specimens, 56 (i e 22 4 per cent) cases of gastric heterotopia with parietal cells were found No statistically significant variation with sex was demonstrated (Table 1)

The prevalence of heterotopia in different diseases is given in Table 2 The frequency was largest in the duodenal and prepyloric ulcer group but a considerable number of gastric cancer and ulcer specimens also disclosed parietal cells in the duodenum The heterotopia exhibited statistically significant variations with the underlying disease

The acid output estimated in meq/h is given in Table 3 The distribution was in good accordance with other studies

TABLE 2 *Prevalence of Parietal Cell Heterotopia According to Gastric Disease Entire series*

Type of resection specimens	No of cases	No of cases with heterotopia
Cancer	120	12 (10 0%)
Gastric ulcer	55	7 (12 7%)
Prepyloric ulcer	23	10 (43 5%)
Duodenal ulcer	52	27 (51 9%)
Total	250	56 (22 4%)

χ^2 = 41 58 df = 3 p<0 0005

TABLE 3 *Acid Output in 203 Resection Specimens*

Type of resection specimens	Acid output (meq/h) Mean	Range
Cancer	6 8	0 0 – 36 8
Gastric ulcer	10 2	0 0 – 30 2
Prepyloric ulcer	28 3	9 8 – 52 4
Duodenal ulcer	32 8	14 2 – 58 4

TABLE 4 *Prevalence of Parietal Cell Heterotopia According to Acid Output, Entire Series Irrespective of Diagnosis*

Acid output	No of cases	No of cases with heterotopia
Achlorhydria	44	1 (2 3%)
0 0–10 0	49	1 (2 0%)
10 1–20 0	34	4 (11 8%)
20 1–30 0	28	12 (42 9%)
Above 30	48	30 (62 5%)
Total	203	48 (23 6%)

$p < 0\ 000\ 000\ 001$

TABLE 5 *Prevalence of Parietal Cell Heterotopia According to Acid Output, Gastric Cancer Specimens*

Acid output (meq/h)	No of cases	No of cases with heterotopia
Achlorhydria	40	1 (2 5%)
0 0–10 0	26	1 (3 9%)
10 1–20 0	14	2 (14 9%)
20 1–30 0	5	2 (40 0%)
Above 30	4	2 (50 0%)
Total	89	8 (9 0%)

$p < 0\ 01$

Table 4 indicates a statistical correlation between the prevalence of gastric mucosal heterotopia and acid output according to rank correlation analysis This correlation is also evident in the individual gastric diseases (Table 5 to 8 inclusive) It is not significant in the duodenal and prepyloric ulcer groups considered separately, but this is most likely due to the relatively small number of cases in these groups, i e to low power of the test If prepyloric and duodenal ulcers are worked up together, statistical significance is obtained (Table 9)

In the duodenal resection specimens, gastric heterotopia was found in five cases. In one of the cases heterotopia was found in both the first and the second part This last mentioned localization consisted of surface epithelium only Acid estimation had been done in only one case, the AHR being 20 8 meq/h

TABLE 6 *Prevalence of Parietal Cell Heterotopia According to Acid Output, Gastric Ulcer Specimens*

Acid output (meq/h)	No of cases	No. of cases with heterotopia
Achlorhydria	4	0 (0 0%)
0 0–10 0	22	0 (0 0%)
10 1–20 0	15	2 (13 3%)
20 1–30 0	6	3 (50 0%)
Above 30	1	1 (100%)
Total	48	6 (12 5%)

$p < 0\ 01$

DISCUSSION

The frequency of gastric heterotopia in this work is in good accordance with previous investigations *Hoedemaeker* (1970) found gas-

TABLE 7 *Prevalence of Parietal Cell Heterotopia According to Acid Output Prepyloric Ulcer Specimens*

Acid output (meq/h)	No of cases	No of cases with heterotopia
Achlorhydria	0	0 (0 0%)
0 0–10 0	1	0 (0 0%)
10 1–20 0	3	0 (0 0%)
20 1–30 0	7	4 (57 1%)
Above 30	9	6 (66 7%)
Total	20	10 (50 0%)

$0\ 10 > p > 0\ 05$

TABLE 8 *Prevalence of Parietal Cell Heterotopia According to Acid Output, Duodenal Ulcer Specimens*

Acid output (meq/h)	No of cases	No of cases with heterotopia
Achlorhydria	0	0 (0 0%)
0 0–10 0	0	0 (0 0%)
10 1–20 0	2	0 (0 0%)
20 1–30 0	10	3 (30 0%)
Above 30	34	21 (61 8%)
Total	46	24 (52 2%)

0 10>p>0 05

tric mucosa with parital cells in the duodenum in 30 per cent of 158 randomly obtained resection specimens Our figures are a little smaller, presumably on account of the relatively large number of specimens with cancer

The figures of *James* (1964) are not directly comparable with our results but demonstrated the same tendency in prevalence for the different groups of diseases

Our results could not confirm the observation of *James* (1964) that the heterotopia is more often seen in women

The above mentioned investigators have found heterotopia to be particularly common in cases with duodenal ulcers We confirmed these observations but found nearly as high a percentage for prepyloric ulcers Since both duodenal and prepyloric ulcers are characterized by a high gastric acid output, it seems reasonable to consider the acid level of importance in the development of the heterotopia

TABLE 9 *Prevalence of Parietal Cell Heterotopia According to Acid Output Prepyloric and Duodenal Ulcer Specimens*

Acid output (meq/h)	No of cases	No of cases with heterotopia
Achlorhydria	0	0 (0 0%)
0 0–10 0	1	0 (0 0%)
10 1 20 0	5	0 (0 0%)
20 1 30 0	17	7 (41 2%)
Above	43	27 (62 8%)
Total	66	34 (51 5%)

p<0 05

Our work has shown that the frequency of heterotopia increases significantly with higher acid output no matter what gastric disease is present On the other hand, a high acid level was not a necessary precondition for the heterotopia Two specimens in the cancer and gastric ulcer groups demonstrating heterotopia had an AHR value below 10 meq/h The average age of these patients was 71 8 years, and it cannot be ruled out that formation of the heterotopic elements had taken place in an earlier period of the patients life, when acid secretion may have been high If this is the case, why the heterotopia persisted, is hard to explain

In so far as a high acid level is of pathogenic importance, the way in which it acts is uncertain The presence of gastric *surface* epithelium in the duodenum could indicate a simple protective response to excessive acid secretion *James* (1963 & 1964) reported about two cases with Zollinger Ellison syndromes, in which gastric surface epithelium was present in the duodenum In this syndrome the acid level of the duodenum is extremely high (*Woodward* 1967) and a protective function seems reasonable Whether significant ulcerative or erosive damage to the original epithelium is in fact essential for the development of heterotopia is unclarified *Florey* and *Harding* (1935) and *Rhodes* (1964) observed heterotopia in the upper duodenum of cats in response to injury

The presence of parietal cells in the duodenum is more difficult to explain The high acid level may cause the duodenal mucosa to differentiate in an abnormal way, as presumed by *Hoedemacker* (1970) or the heterotopia could be one aspect of a basic disorder —namely increased acid secretion If so, it has the same pathogenesis as the increased number of parietal cells in the stomach

The localization of the heterotopia in different parts of the duodenum could only be estimated in the duodenal resection specimens All elements were situated in the first

part with the exception of one case, in which surface epithelium was found in the second part, too This case corresponds to one of the Zollinger-Ellison cases reported by *James* (1963) Our patient had a solid pancreatic tumour, which was not interpreted as an islet cell tumour

In another work (*Johansen* & *Hansen* 1972) we have described the occurrence of several small excresences in the duodenum consisting of gastric mucosa with parietal cells These excresences were nearly all situated in the first part, only a few being in the second This very special localization of the heterotopia could indicate that the phenomenon is not a simple metaplasia Gastric epithelium has been described in several other parts of the intestinal tract The most well-known is Meckel's diverticulum Other localizations are mentioned in the surveys of *Nicholson* (1932) and *Taylor* (1927) In addition to these, heterotopia has been described in enteric duplications (*Grosfeld et al* 1970) In none of these cases has a relation to any physiological condition been demonstrated

The practical importance of the heterotopia is self-evident Duodenoscopy with biopsy has greatly increased, and ignorance of the condition will create many misunderstandings between the endoscopist and the pathologist.

We wish to thank dr *Bjørn Andersen* for assistance with the statistical analysis

REFERENCES

1 *Florey, H W* & *Harding, H E* The healing of artificial defects of the duodenal mucosa. J Path Bact *40* 211–218, 1935

2 *Grosfeld, J L, O'Neill, J A* & *Clatworthy H W* Enteric duplications in infancy and childhood Ann Surg *172* 83–90, 1970

3 *Hoedemacker, P J* Heterotopic gastric mucosa in the duodenum Digestion *3* 165–173, 1970

4 *James, A H* Gastric epithelium in the duodenum of a patient with gastric hyperacidity Proc 2nd Wld Congr Gastroenterology *2* 540 543, 1963

5 *James, A H* Gastric epithelium in the duodenum Gut *5* 285 294, 1964

6 *Johansen, Aa* & *Hansen, O H* Macroscopically demonstrable heterotopic gastric epithelium in the duodenum Scand J Gastroent *8* 59–63, 1973

7 *Marks, J N* & *Drysdale, K M* A modification of Zimmermann's method for differential staining of gastric mucosa Stain Technol *32* 48–50, 1957

8 *Nicholson, G W* Heteromorphoses (metaplasia) of the alimentary tract. J Path Bact. *20* 399–416, 1923

9 *Rhodes, J* Experimental production of gastric epithelium in the duodenum Gut *5* 454 458, 1964

10 *Taylor, A L* The epithelial heterotopia of the alimentary tract J Path Bact *30* 415-449, 1927

11 *Woodward E D* Endocrine induced peptic ulcer In Blakemore W S & Ferguson L.K. (Ed) *Current Perspectives in Gastroenterology* Harper & Row, New York 1967 p 111 124

Acta path microbiol scand Section A 81, 5, 681-697, 1973

FAMILIAL PLASMA LECITHIN: CHOLESTEROL ACYLTRANSFERASE (LCAT) DEFICIENCY

Ultrastructural Aspects of a New Syndrome with Particular Reference to Lesions in the Kidneys and the Spleen

TORSTEIN HOVIG and EGIL GJONE

The University Institute of Pathology, Electron Microscopic Laboratory and Med Dept A, Rikshospitalet, Oslo 1, Norway

Kidney biopsies, plasma, and aspirated spleen specimens from a patient with an inborn error of metabolism, Familial LCAT-deficiency, have been studied by light and electron microscopy The kidney lesions were characterized by deposition of phospholipid membranes and membrane bound particles in the glomeruli and small arteries and veins This material was found within the glomerular capillary lumina, in the capillary wall, in the mesangium and in Bowman's capsule The endothelial cells appeared partly to be detached from the basement membrane which became exposed to blood flow The lipid material was found in the subendothelial space as well as in the basement membrane (both on the endothelial and epithelial side) Arteries and veins showed a similar pattern of lipid deposition Foam cells were frequently seen in the glomeruli as well as in the interstitial tissue It is suggested that the deposition of phospholipid membranes (rich in lecithin and free cholesterol) and lipid particles (possibly with a high content of triglycerides) cause tissue damage and leads to progressive renal failure The spleen contained numerous lipid laden cells which are assumed to be partly responsible for the splenomegaly found in patients with Familial LCAT deficiency Examination of plasma with negative staining technique revealed membranes and membrane-bound particles indicating that such structures are circulating with the blood and may leak out in the vessels and the perivascular tissue

An inborn error of metabolism, called Familial Lecithin Cholesterol Acyltransferase (LCAT) Deficiency was recently discovered in Scandinavia (5, 11, 14, 15) Six living patients in three different families have been described Clinically, the syndrome is characterized by corneal opacity, anaemia and proteinuria In all patients, plasma is turbid or milky and deficient in the plasma cholesterol esterifying enzyme LCAT This enzyme, most likely produced in the liver acts on circulating lipoproteins by catalysing the transfer of fatty acid from the beta position of lecithin to the 3-β OH group of free cholesterol (7) Normally, about 75 per cent of plasma cholesterol circulates in the esterified form, mainly due to the action of LCAT In the patients, low levels of cholesterol ester and lysolecithin in plasma and high levels of unesterified cholesterol and lecithin in plasma and erythrocytes (6), are apparently directly caused by absence of the enzyme Several abnormal plasma lipoproteins have been demonstrated, having abnormal lipid concentration and

Received 20 ii 73 Accepted 21 iii 73

being heterogenous in size Also these lipo protein abnormalities are caused by the LCAT deficiency (8, 16) Advanced renal failure has developed in two patients One died in uraemia at the age of 41 (11) and one, A R (5), has been on haemodialysis since April 1970

No morphological studies of possible tissue alterations in these patients have been re ported, apart from light microscopic de monstration of foam cells in kidneys (5, 15) and sea blue' histiocytes in spleen and bone marrow (12) We therefore have carried out a light microscopical and ultrastructural in vestigation of biopsy material The kidney lesions and some aspects of the morphological alterations in the spleen will be described in the present report In addition, electron mi croscopic studies of plasma with negative staining technique has been carried out in order to establish a possible relationship be tween the plasma alterations and the tissue reactions In view of the findings, some pos sible pathogenetic mechanisms will be dis cussed

CASE REPORTS

A female patient I S age 36 years was studied during her stay in the Medical Department A of Rikshospitalet Oslo in September 1971 She is one of the three originally described sisters with Fa milial LCAT Deficiency (5 14 18) Proteinuria was described in 1955 Since childhood she has suffered from mild but frequent infections— common colds tonsillitis and cystitis Otherwise she has felt healthy She has had a constant protein uria and mild anaemia On admission she had marked corneal opacities slight ankle oedema but normal findings in heart lungs and abdomen The blood pressure was 160/100 mm Hg Laboratory findings Hgb 9 8 g/100 ml RBC 4 mil./cumm WBC 4 900/cumm platelets 258000/cumm FSR 38 mm in one hour serum iron 50 μg/100 ml Se rum total iron binding capacity 240 μg/100 ml serum proteins 6 4 g/100 ml (albumen 2 9 g/100 ml normal serum globulins) Serum electrolytes were normal Urine analysis revealed proteinuria 0 6 g/100 ml specific gravity 1016 Microscopically a few red cells were observed but no casts Serum urea 53 mg/100 ml creatinine 1 3 mg/100 ml Creatinine clearance 60 ml/min inulin clearance 64 ml/min, PAH clearance 382 ml/min Total se rum cholesterol 608 mg/100 ml free cholesterol 538 mg/100 ml triglycerides 822 mg/100 ml phos pholipids total 948 mg/100 ml, free fatty acids 165 micro m/l Plasma LCAT activity was not detectable

MATERIALS AND METHODS

Biopsies

Specimens obtained by kidney biopsy and spleen puncture from patient I S were instantly trans ferred to the fixative, 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer (pH 7 2), and fixed for 2–3 hours The specimens were postfixed in 1 per cent osmium tetroxide in Tyrode buffer (pH 7 2) for 1 hour dehydrated in increasing concentrations of ethanol, and embedded in Epon 812 Semithin and ultrathin sections were obtained with a LKB ultramicrotome The semithin sections were stained with toluidine blue and the ultrathin sections were stained with lead citrate and uranyl acetate

Blood Samples

In
drawn
7 ml
sodium citrate (9 ml of blood to 1 ml of 3 8 per cent sodium citrate) The samples were centrifuged at 200 x G for 10 minutes at room temperature The supernatant was centrifuged for 15 minutes at 800 x G to obtain platelet poor plasma (*PPP*)

A drop of the glutaraldehyde fixed plasma or citrate plasma was placed on a copper grid and stained with phosphotungstic acid (PTA) at pH 7 0

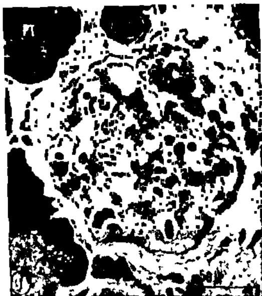
Electron microscopic examination was carried out with a Siemens Elmiskop I at accelerating voltage of 80 KV and 50 μ objective aperture

RESULTS

Kidney Lesions

Light microscopic examination of semithin sections revealed 12 glomeruli which all

(Fig 1) There was usually little or no nar rowing of the capillary lumina, which were devoid of cells or contained some erythrocytes and an occasional granulocyte Within some of the capillary lumina, a darkly stained sub stance was observed (Fig 1) Highly vacuo lated (foam) cells were also observed The capillary basement membrane appeared ir



tubule (PT) The arrows indicate presence of a foreign material Note the prominent mesangial reg ons Epon embedded specimen, semithin section stained with toluidine blue



Fig 3 Foam cells in the interstitial tissue of the kidney Toluidine blue stained epon section

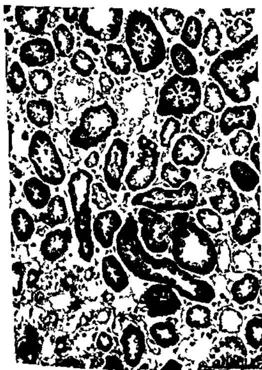


Fig 2 Light micrograph demonstrating different parts of the nephron Same biopsy as in Fig 1 Toluidine blue stained epon section

regularly thickened Proliferation of both parietal and visceral epithelial cells was noted, whereas there appeared to be little or no endothelial cell increase or swelling (Fig 1) The mesangial regions were prominent

The proximal tubular cells were more vacuolated than normally seen (Fig 2) Within the lumen proteinacous material and red blood cells could be observed The distal tubular apparatous revealed no evident alterations

The interstitial tissue showed a slight fibrosis and contained foam cells (Fig 3) often interspersed between the tubules

The small arteries and arterioles were clearly altered with thickening of the intima and narrowing of the lumen (Fig 1)


MES
EP
EP
END
MES
CL
4
10 μ
BC

Electron Microscopy

1 *Glomeruli* The most striking ultrastructural feature of the glomeruli was the presence of electron dense membranes, partly surrounding an amorphous, mottled substance or an electron lucent space These structures were present within the capillary lumina, in the capillary basement membrane, in the mesangial regions, and in the pericapsular areas (Fig 4)

In Fig 5 is demonstrated the presence of such material in the lumen of a glomerular capillary The whole lumen could be occupied by a meshwork of membranes In the lumen of other capillaries, weakly stained particles, partly surrounded by a membrane, were found, as seen in Fig 6 In some of the capillary loops, the endothelial cells had disappeared and the membranous material was observed to be directly associated with the basement membrane (Fig 7) This material could also be found deposited between the endothelial cells and the basement membrane, and the endothelial cells appeared to be partly detached from the basement membrane The endothelial cells in such areas showed morphological alterations with blurring of the details and vacuolization

Deposition of similar material was found in the basement membrane both on the endothelial and epithelial side (Fig 8) In such areas the basement membrane was thickened and electron lucent zones were observed This phenomenon may in part be a preparation artifact due to shrinkage Similar findings were also made in the basement membrane of Bowman's capsule

At a higher magnification the membranes appeared either homogenous or contained 3 or more membranes with a lamellar arrangement similar to that demonstrated in Fig 11 The thickness of the single membrane was about 35 Å and the distance between neighbouring membranes was about 30 Å The central area within a ring of such membranes appeared empty or contained amorphous mottled material

Similar material was frequently found in the mesangial regions both in the matrix and

Fig 4 Electron micrograph of the same biopsy as in Fig 1 Bowman's capsule (BC) capillary lumina (CL) epithelial cells (EP) mesangial cell (MES) The arrows indicate presence of electron dense material within the capillary lumina as well as in other structural parts of the glomerulus Note increase in mesangial matrix and irregular thickening of capillary basement membrane × 2 700



Fig 5 From a capillary loop filled with membrane surrounded particles and membranes Epithelial cell (EP) × 18 000

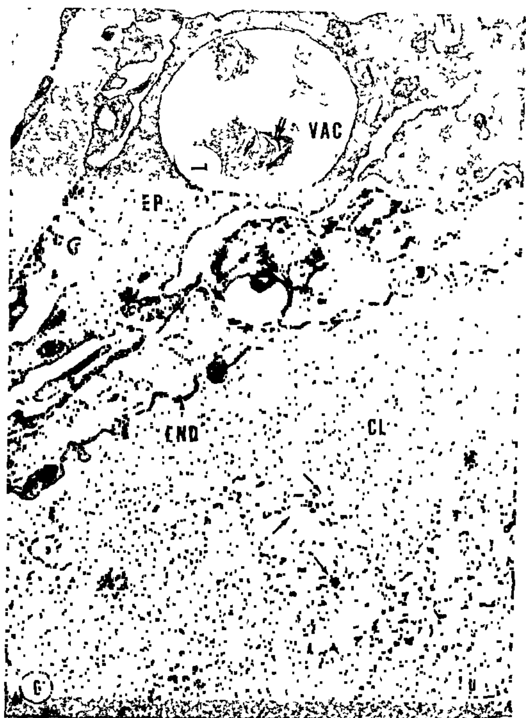


Fig 6 The capillary lumen (CL) is mainly occupied by amorphous material representing plasma proteins, but small particles, partly surrounded by a membrane, can be seen (arrows) Fenestrated endothelium (END) Epithelial cell (EP) with a large cytoplasmic vacuole containing a lipid droplet (L) and some unidentified material (double arrows) Fusion of foot processes Note membrane material in the basement membrane × 15 000

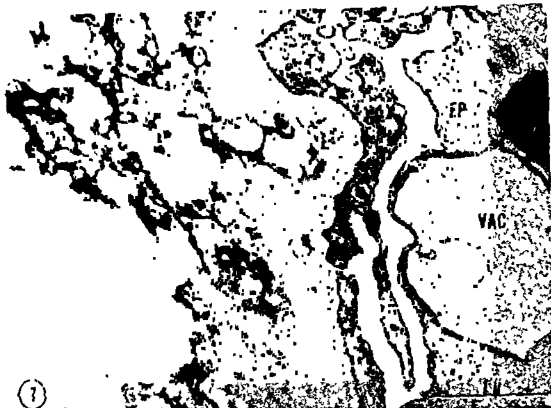


Fig 7 Membranous material in direct contact with the irregular basement membrane (BM) The endothelium *is lacking Fusion of the foot-processes* Epithelial cell (EP) contains a lysosome like structure (L) and a vacuole (VAC) × 30 000

within the cytoplasm of the mesangial cells In such areas there were increased amounts of ground substance, and the mesangial cells had increased in number

Fibrils with a periodic structure (Fig 9) occurred in the mesangial regions, in the capillary lumina, and in vacuoles of epithelial cells The diameter of the fibrils was about 200 Å and the period about 60 Å These fibrils were often seen intermingled with the membrane structures (Fig 9)

An occasional granulocyte was found within the capillary lumen There was no evidence of phagocytosis of pathological membrane structures A single aggregate of three well-preserved blood platelets occurred, but otherwise there was no morphological evidence of thrombosis No fibrin strands occurred

The foot processes were fused in large areas The podocytes contained several vacuoles Within these vacuoles, weakly stained droplets of various sizes and fibrils as described above (Fig 9) could be found Similar alterations were also seen in the parietal epithelial cells

2 *Arteries and veins* Alterations similar to those observed in the wall of the glomerular capillaries were also seen in small arteries and veins The pathological membranous material could be seen underneath the endothelial cells (Fig 10), and the endothelium appeared partly to be detached from the underlying structure Furthermore, an extensive hyalinization of the wall leading to a marked narrowing of the vessel lumen, was noted Membranous structures were observed in all regions of the vessel wall, intracellularly as well as between the cells (Fig 10) As seen in Fig 11, concentrically arranged membranes could be noted Foam cells were

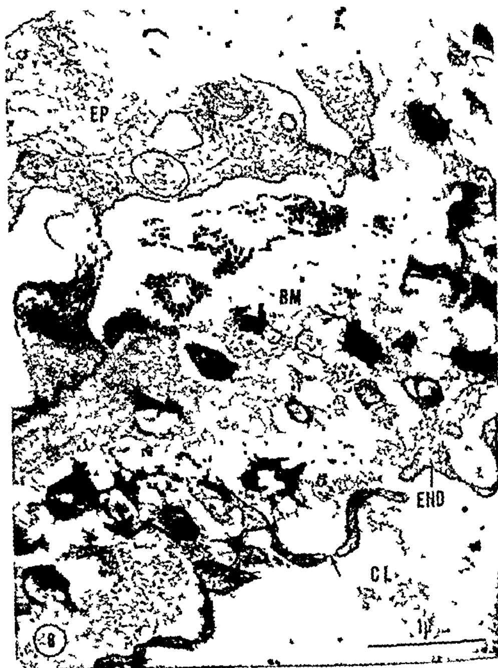


Fig 8 The basement membrane (BM) is largely occupied by membrane-surrounded particles and membranes. The basement membrane shows electron lucent areas and have lost its normal structure. Capillary lumen (CL); endothelium (END) with a diaphragma covering the gap (arrow); epithelial cell (EP). × 30 000

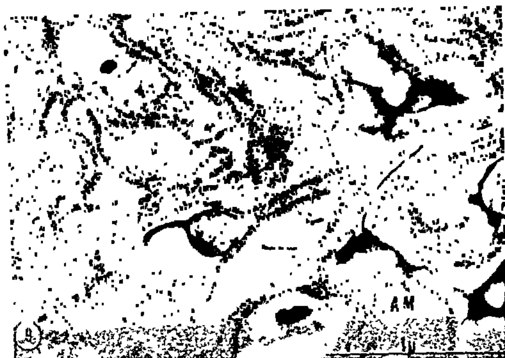


Fig 9 Cross striated fibrils and membranes in a mesangial area The nature and amorphous material (AM) of the fibrils is unidentified × 45 000

also located in the vicinity of the vessel wall

Weakly stained particles, completely or only partly surrounded by a membrane, (Fig 12) could be seen within the vessel lumen Their shape and size appeared to vary, the diameter being within the range of about 500–3000 Å Apart from the surrounding membrane they appeared homogenous No fibrin could be observed in association with these structures, neither was fibrin found in the vessel wall Blood platelet thrombi were not observed

3 *The tubular system* Some of the proximal tubules contained several red blood cells and cellular debris in the lumina The tubular cells, especially in the apical regions, were more vaculated than normally seen Any membranous material was rarely found within these vacuoles, but such material could be seen in the tubular lumen The microvilli usually had a normal appearance

The remaining part of the tubular apparatus did not reveal any striking pathological alterations

4 *The interstitial tissue* A moderate increase of interstitial tissue with fibrotic areas was found There was no accumulation of inflammatory cells, although occasional leucocytes were observed Highly vacuolated ("foam") cells were, however, interspersed in the interstitium, often also located between closely related tubular elements These cells contained numerous droplets of varying size and with a peripheral osmophilic zone (Fig 13), corresponding to the appearance of neutral fat droplets

Spleen Examination of aspirated spleen material revealed a great number of cells containing osmophilic granules, membranous structures and vacuoles in their cytoplasm (Figs 14 and 15) Many of these cells were heavily loaded with these structures and correspond to the so-called "sea-blue" histocytes The granules were composed of concentric membranes with a diameter of about 35 Å (Fig 15) The distance between the membranes was about 30 Å Membranes of similar diameter, could, however, also be found in



Fig 10 This micrograph demonstrates typical changes in a small kidney artery Hyalinization of th vessel wall and lipid deposition (*arrows*), endothelial cells (END) appear to be partly detached, and th vessel lumen (L) is narrowed



Fig 11 Detail from the same vessel as in Fig 10 Note the layered structure of the membrane material (arrow) × 90 000

the cytoplasmic matrix and in the extracellular space

Spleen lymphocytes and granulocytes appeared normal

n

arallel lines in a finger print arrangement Fig 16) The diameter of the membranes was about 30 Å and the distance between hem was about 20 Å Furthermore a mesh work of membranes could be seen (Fig 17) Phosphotungstic acid (PTA) (dark in the print) was found within the meshes, indicating that the meshes were devoid of organic material Particles of various size, however with a mottled appearance and surrounded by membranes were also noted (Fig 17)

H*

DISCUSSION

The presence of membrane bound particles and free membranes in the glomerular capillary lumina and in other structural parts of the glomeruli appear to be specific for Familial LCAT-deficiency As far as the authors are aware, similar findings have not previously been reported The observed membranes and particles in the negatively stained plasma specimens and in vessel lumina in the



Fig 12 Detail from the lumen of a small vein showing partly membrane surrounded particles with somewhat irregular shape and homogenous structure with low electron density × 90 000



Fig 13 Typical foam cell with faintly stained droplets which appear to be in close association with each other (arrows) × 15 000

ultrathin sections support the view that the structures are circulating with the blood stream The great amounts found in the glomerular capillaries may indicate that they are concentrated in these vessels a phenomenon probably related to the large volume of blood passing and filtering through them The possibility cannot be excluded that the pathological material may become trapped in occasional loops exhibiting a thrombus like effect

The nature of the membranes and membrane bound particles is of considerable interest as to the understanding of the mechanisms involved in the kidney damage and the pathological alterations of the spleen in Familial LCAT deficiency The plasma lipoproteins of these patients are known to contain increased amounts of lecithin (phosphatidylcholine) and free cholesterol (8) In electron microscopical studies on isolated fractions of serum or plasma from such patients, the finding of abnormal lipoprotein particles has been reported (3 19) Myelin like structures were, however, not described. According to *Bangham* & *Horne* (1) and *Lucy* & *Glauert* (13) lecithin dispersions may contain spherulites composed of concentric lamellae Preparations of equal molar concentrations of lecithin and free cholesterol were described as being similar to lecithin alone (1)

Hamilton et al (10) and *Seidel et al* (17) have studied serum or plasma from patients with cholestasis and have reported electron microscopic observations of abnormal coin or disc like lipoprotein particles Both groups of investigators concluded that the particles contain a mixture of cholesterol and choline phosphatides probably associated with the wall of the particles Furthermore, it was demonstrated that the particles could be transformed to membranes arranged in a finger print configurations by treatment with phospholipase A_2 or small amounts of lysolecithin (17) These structures would seem to correspond with the membranes we have found in the plasma from our patient We therefore suggest that the membranes found in the plasma from our patient with Familial LCAT deficiency are composed of lecithin (phosphadityIcholine) and free cholesterol, possibly associated with proteins

Forte et al (4) have suggested that the presence of cholesterol esters is required if a normal morphology of plasma high density lipoproteins (*HDL*) is to be maintained Thus the inability to convert plasma unesterified cholesterol to cholesterol ester as seen in patients with LCAT deficiency may

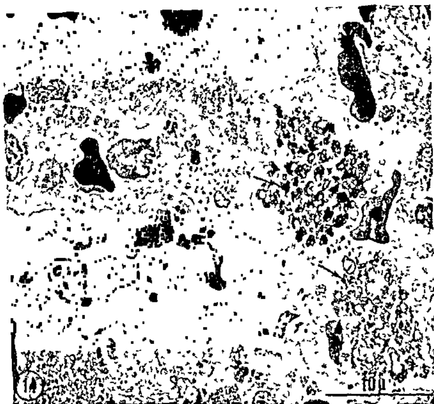


Fig 14 Low power micrograph from aspirated spleen specimen Numerous cells containing large granules and membranes (arrows) × 2 700

be responsible for the pathological appearance not only of the HDL particles, but also of other circulating plasma lipoproteins

Whether the particles we have observed in the tissue specimens correspond to the reported abnormal particles in isolated fractions of serum or plasma (3, 19) is not clear at the moment The electron lucent structures we have found both within and outside the vessels may represent neutral fat As the plasma level of triglycerides is high in patients with LCAT-deficiency, it may be suggested that the central core of the particles may represent triglycerides, the surrounding membrane being composed of lecithin and free cholesterol

The damaging effects of these structures on the glomeruli are pronounced as judged from the present observations The disappearance of the endothelial lining in the glomerular capillaries may be a direct toxic or anoxic effect Another possibility is that if the pathological material is filtered off the vessel lumen through gaps between the endothelial cells and the deposition of the structures in the subendothelial space, it may lead to detachment of the endothelium, exposing the basement membrane to blood constituents The absence of platelet-fibrin thrombi in such areas is surprising, but may be due to a thrombus preventing effect of the lipid material The deposition of the foreign material in the basement membrane, as well as the damage of the endothelium, may be responsible for the proteinuria The damage of the basement membrane is likely to cause reduced filtration efficiency and increasing insufficiency in kidney function Whether the deposited lipid material has any antigenic effects is not known at present, but should be further explored The apparently empty spaces in association with the membranes may reflect tissue shrinkage, but a lytic effect



Fig 15 Detail of a granule demonstrating the concentric arrangement of membranes × 120 000

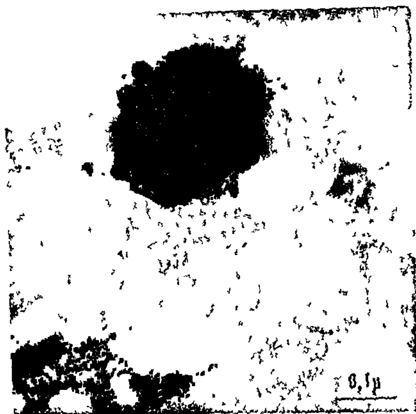


Fig 16 Negatively stained specimen of plasma from patient I S Membranes in a "finger print" arrangement × *160 000*

on the matrix of the basement membrane is also possible

The prominence of the mesangial regions was partly due to the deposition of the material, but the increase in mesangial matrix was also evident The mesangial cells appeared to phagocytize the lipid material a process reflecting the clearance function of these cells (2)

The nature of the cross striated fibrils often located in vacuoles of the visceral epithelial cells is not clear From their appearance it can be concluded that they do not correspond to periodic structures such as fibrin or collagen

The apical vacuolization of the proximal tubular cells may reflect pathological resorption conditions, and some of the vacuoles probably represent variations of multivesicular bodies and cytosegrosomes

The lipid laden cells are probably the main cause of the spleen enlargement The appearance of these cells correspond to those described in our earlier report on sea blue histiocytes (12) The granules with their concentrically arranged membranes may represent break down products of phagocytized cells such as erythrocytes They may however at least in part represent phospholipid containing membranes taken up by the cell It should be emphasized that numerous membranes could be observed in the cytoplasmic matrix This finding may reflect a pathological metabolism of the cell or phospholipid material taken up from plasma The lipid loading of the reticuloendothelial system of the spleen is likely to interfere with its function

As far as the authors are aware there are no previous reports according to which plasma containing pathological lipid particles and membranes were found to cause tissue damage in human disease The present observations therefore may seem to open a new

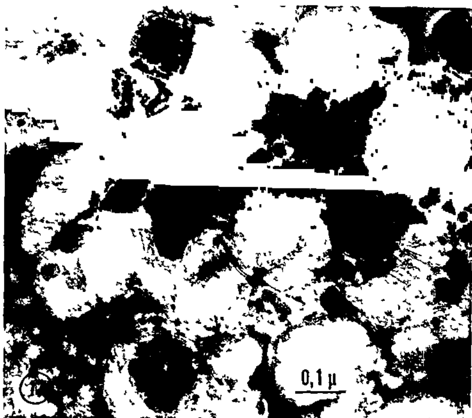


Fig 17 Another area of the same specimen Membranes as well as mottled particles with a peripheral layered membrane can be seen × 120 000

aspect indicating that lipid structures with an abnormal composition should be considered as possible factors leading to vascular and other tissue damage

---

This study was supported by grants from the *Norwegian Research Council for Science and the Humanities* and from *Anders Jahres Research Foundation* The renal biopsies were taken by *Erik Myhre*, M D

REFERENCES

1 *Bangham A D* & *Horne, R W* Negative staining of phospholipids and their structural modification by surfaceactive agents as observed in the electron microscope J Mol Biol *8* 660, 1964

2 *Churg, J* Electron microscopic aspects of renal pathology In Structural basis of renal disease, edited by Lovell Becker E pp 132 196, Huber Medical Division Harper & Row N Y 1968

3 *Forte, T*, *Norum, K R* *Glomset, J A* & *Nichols, A V* "Plasma lipoproteins in Familial LCAT deficiency Structure of low and high density dipoproteins as revealed by electron microscopy" J *of clin Invest 50* 1141-1148 1971

4 *Forte, T* *Nichols, A V*, *Gong, E L* & *Levy R I* Electron microscopic study on reassembly of plasma high density *aporotein* with various lipids Biochem Biophys Acta, *248* 381 386 1971

5 *Gjone E* & *Norum, K R* Familial serum cholesterol ester deficiency *Clinical study of a* patient with a new syndrome' Acta med scand *183* 107–112, 1968

6 *Gjone, E* *Torsvik, H* & *Norum, K R* Familial plasma cholesterol ester deficiency A study of the erythrocytes Scand J clin Lab Invest *21* 327 332, 1968

7 *Glomset, J A* The plasma lecithin Cholesterol acyltransferase reaction *J Lipid Res* *9* 155 167 1968

8 *Glomset J A* *Norum K R* & *King, W*

'Plasma lipoproteins in Familial LCAT deficiency Lipid composition and reactivity in vitro J of clin Invest *49* 1827 1837, 1970

9 *Glomset, J A, Norum, K R, Nichols, A V, Gjone, E* & *King, W* Evidence for abnormal disposal of chylomicrons in Familial LCAT deficiency J Clin Invest In press

10 *Hamilton, R L, Havel, R J, Kane, J P, Blaurock A E* & *Sata, T* Cholestasis Lamellar structure of the abnormal human serum lipoprotein Science *172* 475–478, 1971

11 *Hamnstrøm B, Gjone, E* & *Norum, K R* Familial plasma lecithin Cholesterol acyl transferace deficiency The Brit Med J *2* 283–286 1969

12 *Jacobsen, C D, Gjone, E* & *Hovig, T* 'Sea blue histiocytes in Familial LCAT deficiency Scand J Hematology *9* 106–113 1972

13 *Lucy, J A* & *Glauert, A M* Structure and assembly of macromolecular lipid complexes composed of globular micelles J Mol. Biol *8* 727, 1964

14 *Norum, K R* & *Gjone, E* Familial plasma lecithin Cholesterol acyltransferase deficiency *Biochemical study of an inborn error of meta*bolism Scand J clin Lab Invest *20* 231–243, 1967

15 *Norum, K R, Glomset, J A* & *Gjone, E* Familial lecithin Cholesterol acyltransferase deficiency In Stanbury, Wyngaarden and Fredrickson The metabolic basis of inherited disease III Edition McGraw Hill NY 531 544, 1972

16 *Norum, K R Glomset, J A, Nichols, A V* & *Forte T* 'Plasma lipoproteins in Familial LCAT deficiency Physical and chemical studies of low and high density lipoproteins. J of clin Invest *50* 1131 1140, 1971

17 *Seidel, D, Agostini, B* & *Muller, P* Structure of an abnormal plasma lipoprotein (LP X) characterizing obstructive jaundice Biochem *Biophys Acta 260 146–152, 1972*

18 *Torsvik, H, Gjone, E* & *Norum, K R* Familial plasma cholesterol ester deficiency Clinical studies of a family' Acta med scand *183* 387 391, 1969

19 *Torsvik, H, Solaas, M H* & *Gjone, E* Serum lipoproteins in plasma lecithin cholesterol acyltransferase deficiency, studied by electron microscopy Clinical Genetics, *1* 139 *150, 1970*

Acta path microbiol scand Section A 81, 5, 698-702, 1973

HISTOLOGICAL CHANGES IN TWO SEROLOGICALLY DEFINED GROUPS OF CHRONIC HEPATITIS

PER CHRISTOFFERSEN, ODD DIETRICHSON, JENS O NIELSEN and PREBEN ELLING

Department of Pathology and Medical Department 2, Kommunehospitalet and the University Clinic for Infectious Diseases, Blegdamshospitalet, Copenhagen, Denmark

Forty two consecutive patients with biopsy verified chronic hepatitis and available serum specimens were divided into two groups group 1 12 patients with persistent Au antigenemia but neither antinuclear antibodies (ANA) nor smooth muscle antibodies (SMA) and group 2 27 patients without detectable Au antigen but with ANA and/or SMA The histological features in the two groups have been compared There is a significantly higher incidence of parenchymal inflammation and a significantly higher degree of portal inflammation in group 2 than in group 1, just as there is more pronounced occurrence of plasma cells. A morphological follow up of 92 per cent of the patients shows a significantly more frequent development of cirrhosis in the group with ANA and/or SMA

Recently *Dietrichson et al* (6) have demonstrated the prognostic relevance of the serological classification introduced by *Wright* (10), *Vischer* (9) and *Bulkley et al* (3) in chronic hepatitis Forty-two consecutive cases with a histologically verified chronic hepatitis and with serum available for examination were divided in two groups *Group 1* with persistence of Australia antigen (Au-antigen) but without either antinuclear antibodies (ANA) or smooth muscle antibodies (SMA) and *group 2* without Au-antigen but with either ANA and /or SMA

Group 1 (with Au-antigen) consists predominately of young males with a history of serum hepatitis while most of the patients in group 2 (with autoantibodies) were middle aged women with an insidious onset of the disease

The activity of the liver disease was greater in the group of patients with auto-antibodies and the prognosis seems to be worse as compared with the Au-antigen positive patients.

The purpose of this work has been to investigate the histological changes in the two groups and whether there are morphological differences between liver biopsies with chronic hepatitis in the two groups

MATERIAL AND METHODS

The material consists of a total of 42 patients all with histologically verified chronic hepatitis (1) Cases morphologically giving suspicion of cirrhosis have been excluded and so have cases with acute viral hepatitis with slight or moderate periportal changes in the form of piece meal necroses.

The material has been selected as consecutive pa

Received 2 iii 73 Accepted 2 iii 73

Requests for reprints should be addressed to *Per Christoffersen* Patologisk Anatomisk Institut Kommunehospitalet, 1399 Copenhagen Denmark

TABLE 1 *Results of Serological Tests*

Serological test	Number of patients examined	Number of patients with positive sera
Au ag	42	13
ANA	42	21
SMA	42	20
MA	42	0

(Au ag – Australia antigen ANA = antinuclear antibodies SMA smooth muscle antibodies MA = mitochondrial antibody)

TABLE 2 *Correlation between Australia Antigen (Au) and Antinuclear Antibodies (ANA) and Smooth Muscle Antibodies (SMA)*

	Au (+) 13 patients	Au (—) 29 patients
ANA (+)	0	21
ANA (—)	13	8
SMA (+)	1	19
SMA ( )	12	10

tients with chronic hepatitis and available serum specimen admitted to Medical Department 2 Kommunehospitalet and The University Clinic for Infectious Diseases Blegdamshospitalet, Copenhagen in the period December 1965- May 1970

The liver biopsies have been performed by the method of Menghini and the tissue has been fixed in neutral formalin and imbedded in paraffin The [illegible] long [illegible] med without knowledge of the clinical data by one of the authors (P C) on hematoxylin and eosin and v Gieson Hansen preparations For all biopsies further sections stained for reticulum fibres (7) iron (8) and pyroninopful substance (2) have been available

The following qualities have been semiquantitatively registered (0 + ++ +++) focal necroses *confluent* necroses piece meal necroses variation *in size of* liver cells and nuclei acidophilic bodies Kupffer *cell proliferation* cholestasis (non-controversial intra and extracellular bile *thrombi*) adenomatous liver cell proliferation fatty *change* parenchymal inflammation passive septa (5) *as well* as iron and lipofuscin deposits in liver cells Kupffer cells and *connective tissue*

Further *the* degree *of* bile duct proliferation fibroses and inflammation *in* portal tracts have been registered Also the occurrence of bile ducts with abnormal epithelium has been recorded

The *clinical* data and the results of the *biochemical* tests have been described in a previous paper (6)

From all patients sera drawn simultaneously with the biopsies were available for *immunological* studies The methods for demonstration of Au antigen, ANA, MA and SMA have been published elsewhere (6)

The material is divided into two groups *Group 1* including patients with Au antigen, but neither ANA nor SMA, and *group 2* consisting of patients without Au antigen, but with ANA and/or SMA

The histological features in the two groups have been compared

On all patients with the exception of three from group 2 *repeat biopsies* have been performed These biopsies have been assessed according to the same criteria as the primary biopsies The average period of observation has been 23 months varying from one to 45 months Twelve patients (all from group 2) have after the initial biopsy received steroids five immunosuppressive treatment and four both steroids and immunosuppressive treatment.

For the *statistical* assessment the chi²— test has been used The limit for type 1—error (2α) has been set to 0 05

RESULTS

Serological Findings

The results of the serological findings are seen from Table 1 Thirteen patients were positive for Au antigen while ANA and SMA were found in 21 and 20 patients respectively MA could not be demonstrated in any of the patients in this study

Table 2 gives the correlation between Au-antigen and circulating autoantibodies (ANA and SMA) None of the patients had both Au antigen and ANA, and only one had simultaneous occurrence of Au antigen and SMA (in low titre) *Group 1* thus consists of 12 patients

ANA and/or SMA were demonstrated in 27 patients without Au antigen and two patients had neither Au antigen nor ANA or SMA *Group 2* thus consists of 27 patients

Morphological Findings

A summary of the *parenchymal* changes in the two groups are shown in Table 3 Apart

TABLE 3 *Distribution of Parenchymal Changes in Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients without Au-antigen but with SM 1 and/or ANA)*

Parenchymal changes	Group 1				Group 2				Differences between group 1 and 2 (chi²-test)
	0	+	++	+++	0	+	++	+++	
Focal necroses	0	10	2	–	1	25	1	–	no significance
Confluent necroses	10	1	1	–	22	2	3	–	no significance
Passive septa	9	1	2	–	20	6	1	–	no significance
Piece meal necroses	3	5	4	–	3	11	12	1	no significance
Acidophilic bodies	5	6	1	–	6	18	3	–	no significance
Variation of cells and nuclei	1	7	4	–	2	17	8	–	no significance
Fatty change	11	1	–	–	18	9	–	–	no significance
Kupffer cell proliferation	0	10	2	–	0	21	5	1	no significance
Cholestasis	12	–	–		24	3	–	–	no significance
Parenchymal inflammation	8	4	–	–	7	20	–	–	$p<0.05$
Lipofuscin in liver cells	0	12	–	–	0	26	1	–	no significance
Iron in liver cells	12			–	25	2		–	no significance
Iron in Kupffer cells	12	–	–	–	22	2	3	–	no significance

TABLE 4 *Distribution of Portal Changes in Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients without Au antigen but with SMA and/or ANA)*

Portal changes	Group 1				Group 2				Differences between group 1 and 2 (chi²-test)
	0	+	++	+++	0	+	++	+++	
Fibroses	0	6	6		0	17	10	–	no significance
Inflammation	0	4	4	4	0	2	7	18	no significance*
Bile duct proliferation	0	9	3		0	16	11	–	no significance
Atypical Bile duct epithelium	12	–	–		20	7	–	–	no significance
Iron in portal macrophages	12			–	19	5	3	–	no significance
Germinal centres	12		–	–	26	1	–	–	no significance

* no difference in the occurrence of portal inflammation but significantly more cases in group 2 with moderate or severe inflammation ($p<0.05$)

from the greater incidence of parenchymal inflammatory cells in group 2 there is no statistically significant difference between the two groups neither as regards incidence nor degree of the registered histologic features

The lobules in both groups in most cases showed slight to moderate changes as in viral hepatitis (focal necroses acidophilic bodies, variation of size of cells and nuclei and Kupffer cell proliferation) About one fifth (7/39) of the cases showed areas of *confluent necrosis* These were most often small and in the centrilobular zone Approximately one fourth (10/39) contained so-called *passive septa* confluent necroses and passive septa occurred simultaneously in about one eighth (5/39) of the specimens

The periportal areas in most cases (33/39) exhibited more or less pronounced *destruction* of the limiting plate with many closely packed often rather small areas of *piecemeal necrosis* The predominant inflammatory cell in the piecemeal necrosis was the plasma cell There are three cases without piecemeal ne

TABLE 5 *The Table shows the Distribution of Repeat Biopsies According to Histological Diagnosis*

Chief histological diagnosis	Group 1 (repeat biopsies from 12 patients)	Group 2 (repeat biopsies from 24 patients)
Cirrhosis	1 (8%)	8 (34%)
Suspicion of cirrhosis		2 (8%)
Chronic aggressive hepatitis	5 (42%)	11 (46%)
Chronic persistent hepatitis	6 (50%)	3 (13%)

crosis (chronic persistent hepatitis) in both groups The chronic aggressive hepatitis is graded as moderate or severe on the basis of the number of areas of piecemeal necrosis There is no significant difference between the groups with regard to activity

Cholestasis and *iron deposits* in liver and Kupffer cells are only demonstrated in a minority of the biopsies and exclusively in biopsies from group 2, but the difference is not statistically significant Significant *steatosis* was absent, expect for a few lipid droplets in approximately one fourth (10/39) predominantly from group 2

The incidence of *parenchymal inflammatory* cells is greater in group 2 than in group 1 ($p < 0.05$) The predominant inflammatory cell in both groups is the plasma cell

A summary of the *portal changes* in the two groups are shown in Table 4

Portal *fibrosis*, *inflammation*, and slight to moderate *bile duct proliferation* are constant in both groups There is no difference in the occurrence of portal *inflammation* in the two groups but there are significantly more cases in group 2 with moderate or severe inflammation than in group 1 ($p < 0.05$) The predominant inflammatory cell is the plasma cell and as a rule their *number* in group 2 is so high that the periportal area appeared as a red zone in pyronine stained sections

Atypical bile duct epithelium is found in seven biopsies exclusively from group 2, but the difference is not statistically significant The epithelial cells of the affected portal bile ducts are swollen Their cytoplasm often looked empty, possible vacuolated, but sometimes it had an eosinophilic granular appearance The cells are more rounded than normal, sometimes being slightly polygonal In other areas the cellular limits are blurred and the nuclei hyperchromatic *The* epithelium is nearly always multilayered, frequently having four to six prominent layers The epithelium and basement membrane are often slightly infiltrated by lymphocytes and sometimes by plasma cells and granulocytes

Germinal centers are only demonstrated in one biopsy and are here in close relationship to the parts of the biliary tree exhibiting abnormal epithelium

Iron deposits in portal macrophages are only demonstrated in group 2 (8/27), but the difference is not statistically significant

Follow Up

Table 5 shows the distribution according to chief histological diagnosis of the repeat biopsies in group 1 and 2 One case (8 per cent) from group 1 developed cirrhosis The last 11 cases from this group still show chronic hepatitis (6 with chronic persistent and 5 with chronic aggressive)

Eight (34 per cent) of the 27 patients in group 2 have developed cirrhosis and in two cases there is a suspicion of cirrhosis. The last 14 cases still show chronic aggressive hepatitis (11) and chronic persistent hepatitis (3) None of the repeat biopsies in this group showed histological improvement There are significantly more repeat biopsies with cirrhosis in group 2 than in group 1 ($p < 0.001$)

DISCUSSION

In chronic hepatitis one may in the light microscope observe liver cells disintegrating in the following manners focal necroses, confluent necroses, piece meal necroses and acidophilic bodies If the histological activity in a chronic hepatitis is expressed by the amount of disintegrated liver cells, especially the

piece-meal necroses and the inflammatory reaction brought about by these there is, in the presented material, no differences in the two groups with regard to incidence and degree of liver cell disintegration, but the incidence of parenchymal inflammation and the degree of portal inflammation were significantly greater in the group with autoantibodies The predominant inflammatory cell was in both parenchyma and portal tracts the plasma cell, and as a rule, their number in the group with autoantibodies was so high that the periportal area appeared as a red zone in pyronine stained sections

The above mentioned is in good agreement with the biochemical findings (6) with significantly elevated values of Ig A and Ig G in the group with autoantibodies and with the suggestion that the chronic hepatitis in this *group is a autoimmune disease*

It is remarkable but not statistically significant, that iron deposits in liver cells, Kupffer cells, and histiocytes are only demonstrated in the group with autoantibodies At the present time our knowledge about iron deposits in acute and chronic hepatitis is too sparse to allow any evaluation of the finding mentioned

Abnormal bile duct epithelium is only demonstrated in the group without Au antigen and this finding is in accordance with earlier findings (4) It has been suggested that chronic hepatitis with abnormal bile duct epithelium is an expression of a more pronounced effect of auto-immunological factors contributing *to the pathogenesis and causing* a graver prognosis This suggestion is an accordance with the results in this investigation as four of the eight cases of cirrhosis found in the follow-up study in the group with autoantibodies developed in cases with abnormal bile duct epithelium

The comparative histological study thus give some support to the suggestion of at least two different types of chronic hepatitis, and may reflect pathogenic differences between the two groups

REFERENCES

1 *Bianchi, L , de Groote, J , Desmet, V , Gedigk, P Korb, G , Popper, H , Poulsen, H , Scheuer, P J , Schmid, M , Thaler, H & Wepler, W* Morphological criteria in viral hepatitis Lancet i 333–337, 1971

2 *Brachet, J* La Localization des acides pentosenucliques dans les tissue animaux et les aufs d Amphibiens en voie de development Arch de biol *53* 207–219, 1912

3 *Bulkley, B Heizer, W D , Goldfinger, S E Isselbacher, K J & Schulman, N R* Distinctions in chronic active hepatitis based on circulating *hepatitis-associated antigen.* Lancet ii 1323–1326, 1970

4 *Christoffersen, P , Dietrichson, O , Faber, V & Poulsen, H* The occurrence and significance of abnormal bile duct epithelium in

5

G , Popper, H , Poulsen H , Scheuer, P J , Schmid, M Thaler H Uehlinger, E & Wepler W A classification of chronic hepatitis Lancet ii 626–628 1968

6 *Dietrichson O , Nielsen J O , Elling, P & Christoffersen P* The relevence of a serological classification of chronic hepatitis Acta path microbiol scand Section B (in press)

7 *Gomori G* Silver impregnation of reticulum in paraffin sections Am J Path *13 993 1001 1937*

8 *Perls M* Nachweiss von Eisenoxyd in gewissen Pigmenten Arch Pathol Anat *Phys 39* 42–48 1867

9 *Vischer, T L* Australia Antigen and Autoantibodies in Chronic Hepatitis British Medical Journal *2, 695–698 1970*

10 *Wright R* Australia antigen and smooth muscle antibody in acute and chronic hepatitis Lancet i 521 522 1970

Acta path microbiol scand Section A 81, 5, 703–714, 1973

MORPHOMETRIC STUDIES OF THE SERTOLI CELLS IN ELDERLY MEN WITH SPECIAL REFERENCE TO THE HISTOLOGY OF THE PROSTATE

An Analysis in an Autopsy Series

THORSTEIN B HARBITZ

Ullevål Hospital, Department of Pathology, University of Oslo, Norway
Head Kristen Arnesen, M D

An index (S_1) for the absolute number of Sertoli cells, based upon the count of Sertoli cell nuclei, was determined in a consecutive autopsy series of 141 men over 40 years of age This investigation formed part of a comprehensive study of morphological characteristics of the testes, the pituitary gland and the adrenal glands in men with prostatic hyperplasia and neoplasia Patients with clinically manifest carcinoma of the prostate or who had previously been subjected to prostatic surgery were not included S_1 varied widely at all ages and within all groups of prostatic histology At both simple and multiple regression analyses, S_1 showed a negative and statistically significant correlation to age The number of Sertoli cells *per se* was not clearly related to the histology of the prostate In men with normal histology of the prostate, who were all under 70 years of age, S_1 remained practically unaltered with advancing age Declining Sertoli cell numbers with age were demonstrated in patients with benign nodular hyperplasia either alone (BNH) or together with carcinoma (C + BNH), although the correlation was statistically significant only within the BNH group

Alterations in the balance between androgenic and oestrogenic hormones with increasing age are commonly assumed to be of importance for the development of prostatic hyperplasia and neoplasia (*Tetlum* 1950, *Moore* 1952, *Geissendörffer* 1960, *Isurugi* 1970) Morphometric studies of steroidogenic tissues in the testes of men at various ages and with abnormal growth of the prostate is therefore of interest

Sertoli cells and Leydig cells constitute the two main sites of testicular hormone production The endocrine function of the Sertoli cell is less well known than that of the Leydig cell which is a long-accepted source of androgenic hormones The view that Sertoli cells produce steroid hormones seems well founded, but it is not clear whether these cells in man produce oestrogens or androgens or both There are strong indications that the Sertoli cell in man is capable of producing oestrogenic hormones (*Berthrong et al* 1949, *Hanafy et al* 1972), although it may not be the only source of oestrogen production in the human testis (*Hooker* 1970, *Johnsen* 1970, *Lacy & Pettitt* 1970) On the other hand, recent reports by *Lacy & Pettitt* (1970) and

Received 1 iii 73 Accepted 1 iii 73
Requests for reprints should be addressed to *Department of Pathology Ullevål Hospital, Oslo 1*

Bell et al (1971) conclude, from experiments in rats, that Sertoli cells have a capacity for elaboration of androgens that is equal to or greater than that of the Leydig cells

Since the appearance of the reports by *Huggins & Hodges* (1941) and *Huggins et al* (1941a, b) on the suppressive effect of oestrogens on prostatic carcinoma, the role played by oestrogenic hormones in abnormal growth of the prostate in elderly men has been eagerly investigated The conclusions to be drawn from the studies that have appeared to date, however, are not clear Histochemical studies (*Lynch & Scott 1950, Tetlum 1950*) have revealed a relative predominance of the lipid content of Sertoli cells over that of Leydig cells with increasing age According to *Tetlum*, this observation gives support to the theory that hyperplastic growth of the prostate in elderly men is related to a relative oestrogen preponderance *Sommers* (1957), on the basis of morphological studies of certain endocrine glands, claimed that an excess of oestrogens was of importance for the development of benign hyperplasia and sometimes for carcinoma of the prostate On the other hand, incidence figures of prostatic hyperplasia and carcinoma in men with liver cirrhosis and signs of hyperoestrogenism do not vary from those in subjects without liver cirrhosis (*Robson* 1966) Finally, hormone assay studies have unveiled lowered urinary concentration of androgens and disturbed oestrogen excretion with low oestradiol concentration in the urine of patients with clinically manifest prostatic carcinoma (*Stern et al* 1964, *Marmorston* 1966)

The present quantitative analysis of Sertoli cells forms part of a comprehensive autopsy study which has been performed to investigate the association between pathological growth of the prostate and morphological changes in the testes, the pituitary gland and the adrenal glands in elderly men Several factors may interfere with the morphological characters of endocrine glands and disturb their usefulness as a measure of endocrine function in man Hence, single variable and multiple regression analyses, including a number of confounding variables, were applied to analyse the relations between prostatic histology and the number of Sertoli cells

MATERIAL AND METHODS

The testes and prostates were collected from 201 consecutive autopsies of men over 40 years of age who died in Ullevål Hospital during a 3 months period 1967 68 Five patients were not suited for further analysis (see *Harbitz* 1973a), and 40 cases were excluded due to autolytic changes in the tissue at hand, making the recognition of Sertoli cells uncertain Patients who had been subjected to prostatic surgery (17), treatment with oestrogenic hormones (3) or both (1) were analysed separately The main analysis of Sertoli cell quantity was therefore based on 141 cases

The techniques for fixation and microscopical

TABLE 1 *Histology of the Prostate by Age in 141 Patients*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	1	0	2	0	0	0	0
50–59	11	3	10	2	0	4	0
60–69	7	1	19	10	3	4	1
70–79	0	1	24	17	0	3	0
80 +	0	0	7	9	0	2	0
Total	19	5	62	38	3	13	1

N = normal histology DA = diffuse atrophy, BNH = benign nodular hyperplasia C = carcinoma, AGP = atypical glandular proliferation

examination of the prostate have been described in a previous report (*Harbitz & Haugen* 1972) The presence of normal histology (N), benign nodular hyperplasia (BNH), carcinoma (C), atypical glandular proliferation (AGP) or diffuse atrophy (DA) of the prostate was noted for each gland The occurrence of atypical glandular proliferation was not specified in glands where carcinoma was diagnosed The histological findings in the prostate of the 141 cases included in the main analysis are presented in Table 1

Sertoli Cells

As described previously (*Harbitz* 1973b), three total sections were prepared from the upper, middle, and lower parts of each testis The sections were stained with phosphotungstic acid haematoxylin

cell (1968) Their nuclei have an oval shape, contain a large nucleolus, and are usually lying at some distance from the basement membrane with the long axis directed radially

The number of Sertoli cells was counted within *the outer limits of the projection of an ocular* square-ruled network which measured 0 33 mm × 0 33 mm serving as the area unit Only cells *with an intact characteristic nucleolus were* counted

The Sertoli cells were counted in four square area units from *each section positioned so as to* represent four concentric areas of equal size, as escribed previously for the quantitation of Leydig ells (*Harbitz* 1973b) Thus a total of 12 fields as counted in each testis* If *c* is the weight of the estis tissue present in a histological section within ne square unit, if *W* is the total weight of the estis and *n* the mean number of Sertoli cells per quare area unit then the total number of Sertoli cells in the testis is $n \times \frac{W}{c}$ *As c can be considered* constant, the total number of Sertoli cells per whole testis was measured by the product n x W, which *was called the Sertoli cell index* (S_1) W was taken in grams

Clinical Data

Clinical data were recorded from the clinical notes and prepared for computer analysis

* In two testes weighing less than 5 grams each only the sections from the upper and lower halves were counted Only the equatorial sections were counted in a case in which the testes weighed approximately 1 gram each. In the smallest sections (115 sections from 7 cases) counting of Sertoli cells was performed in three instead of four visual fields from each section

Statistical Analysis

Modified Student's t tests accounting for unequal variances and numbers of individuals were used for testing differences between arithmetic means and for testing differences between slopes of regression lines (*Snedecor & Cochran* 1967) n_A and n_B being the number of observations in the groups to be compared, p values were based on the least of n_A-1 and n_B-1 degrees of freedom for means, and on the least of n_A-2 and n_B-2 degrees of freedom *for slopes. P values below* 0 05 were regarded statistically significant

Adjustment for age differences was performed *according to the indirect method of standardization* (*Armitage* 1971), using the age specific mean Sertoli cell index of the main series of 141 patients *as standard values*

Multiple regression analysis Stepwise and full multiple regression analyses were applied as previously described (*Haugen & Harbitz* 1972, *Harbitz* 1973a), using Sertoli cell index (X_1) as the *dependent* variable The following factors, either bivariate (1,0, labelled X_2 to X_{12}) or continuous (labelled X_{13} to X_{15}), were treated as *explanatory* (independent) variables

GP)

X_4 Carcinoma (C)
X_5 Diffuse autrophy (DA)

Cause of Death

X_6 Cardiovascular disease*
X_7 Malignant tumour

Duration of Final Illness

X_8 1 7 days
X_9 >7 days

Other

X_{10} Steroid hormone treatment**
X_{11} Diabetes mellitus
X_{12} Liver cirrhosis
X_{13} Age
X_{14} Body weight
X_{15} Body length

* *Includes death from myocardial infarction* (45 cases), cerebrovascular and peripheral vascular disease (14 + 6 cases), rheumatic valvular disease (4 cases), *miscellaneous cardiovascular disorders* (9 cases)

** Other than oestrogenic hormones Includes treatment with corticosteroids (5 cases), anabolic steroids (nortestosterone 3 cases), or both (8 cases)

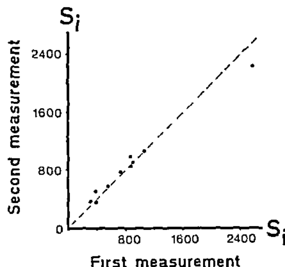


Fig 1 Reproducibility for Sertoli cell index (S_i) estimation *in 10* cases examined on *two occasions*



Fig 2 Cross section of a seminiferous tubule Sertoli cell nuclei (arrow) are distinguishable from the nuclei of cells of the seminiferous series Phosphotungstic acid haematoxylin (PTAH) stain × 235

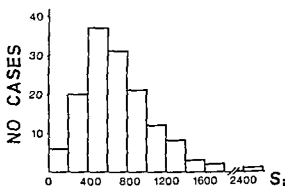


Fig 3 Distribution of Sertoli cell index (S_i) at autopsy in the main series of 141 men aged 40 years or more

Initially, forward stepwise regression analysis was run until all explanatory variables which were partially significant at the 5 per cent level at each step were included Thereafter, the selected variables, together with all groups of prostatic histology (X_2 X_5), were included in the full multiple regression analysis Regression coefficients were calculated according to the method of least squares. Differences between regression coefficients for the various groups of prostatic histology were tested by an F test (*Scheffé* 1959)

The analysis was based on a standard programme for multiple regression (NRSR) developed at The Norwegian Computing Center, Oslo, and was conducted on a Univac 1108 computer

Reproducibility The reproducibility of the method for determining the Sertoli cell index was tested by duplicate estimations in 10 randomly selected cases, as previously described (*Harbit* 1973b) The pairs of observations are compared in Figure 1 The standard deviation of a single observation i e the method error, was 83 7, which is 11 8 per cent of the total mean Sertoli cell index

RESULTS

The histological identification of Sertoli cells was generally distinct The oval, but sometimes irregular, nucleus with a thin membrane and a prominent nucleolus was characteristic (Fig 2) The longitudinal fibrils so often described in the cytoplasm of Sertoli cells were seldom recognizable

The frequency distribution of the Sertoli cell index showed a positive skewness as compared with the normal distribution (Fig 3) Individual observations ranged from zero up to 2558, with a median of 666 (Fig 4a)

The Sertoli cell index in the main series of 141 men decreased gradually with age although the correlation coefficient was low ($r = -0.232$ $p<0.01$) At all ages the index varied over a wide range and the standard deviations within the 10 year age groups were high (Table 2)

The Sertoli cell index was studied in relation to the cause of death duration of the final illness treatment with steroid hormones other than oestrogens clinically manifest diabetes mellitus and liver cirrhosis (Table 3) Patients dying from malignant tumours had higher mean Sertoli cell index than those dying from cardiovascular disease or other causes

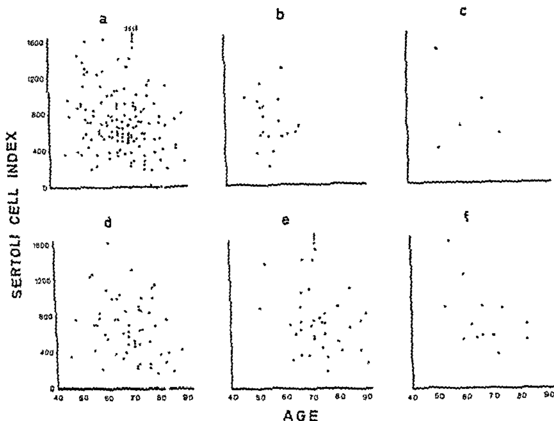


Fig 4 Sertoli cell index plotted against age in the main series of 141 men over 40 years of age (a), and in subgroups of cases with normal histology (b), diffuse atrophy (c), benign nodular hyperplasia (d), carcinoma with (●) or without (O) benign nodular hyperplasia (e), and atypical glandular proliferation with (●) or without (O) benign nodular hyperplasia (f) of the prostate

but the differences were not statistically significant ($p > 0.10$) Correction for age differences between groups did not alter the mean index for the individual cause-of-death groups substantially The duration of the final illness apparently affected the quantity of Sertoli cells very little, although a slight increase in the number of Sertoli cells was indicated when age differences between groups were accounted for The mean Sertoli cell index in subjects who had received steroid hormone treatment was substantially higher than that applying to the whole series, but the standard deviation was high On the other hand the mean Sertoli cell indices in patients with diabetes mellitus and liver cirrhosis were lower than that of the total material

The age specific and total mean Sertoli cell indices within groups of prostatic histology are presented in Table 4 The highest age-specific means were consistently observed in patients with prostatic carcinoma accompanied by benign hyperplasia No matter the age of patients, the Sertoli cell indices were not significantly different in the various groups of prostatic histology Decreasing Sertoli cell indices with advancing age were apparent both in patients with benign nodular hyperplasia alone and in those with carcinoma of the prostate

The scatter of the Sertoli cell index within histological groups is presented in Figures 4b-4f Individual observations showed a wide variation within all groups and at all ages, although the scatter was apparently reduced

TABLE 2 *Sertoli Cell Index in Men Previously not Subjected to Prostatic Surgery or Treatment with Oestrogenic Hormones*

Age	No patients	Sertoli cell index		
		Mean	S D	Range
40–49	3	691 0	305 4	353– 947
50–59	30	800 5	379 1	190–1606
60–69	45	737 0	285 0	310–1627
70–79	45	691 7	438 4	3–2558
80 +	18	526 9	274 1	0–1103
All	141	708 3	364 1	0–2558

S D Standard deviation

TABLE 3 *Sertoli Cell Index and Cause of Death, Duration of Final Illness, Steroid Hormone Treatment, Diabetes Mellitus and Liver Cirrhosis*

	No patients	Sertoli cell index		
		Observed mean	S D	Age adjusted mean
Cause of death				
Cardiovascular disease	78	674 3	315 7	675 7
Malignant tumour	30	832 7	514 1	818 8
Other causes	33	665 0	288 4	672 3
	141			
Duration of final illness				
<1 day	36	676 2	229 5	662 5
1 7 days	32	721 6	347 2	697 3
>7 days	73	714 6	422 5	733 5
	141			
Other				
Steroid hormone treatment*	16	950 1	576 5	936 2
Diabetes mellitus	6	594 2	245 1	610 1
Liver cirrhosis	4	540 3	287 1	497 7
All	141	708 3	364 1	

S D Standard deviation
* Other than oestrogenic hormones

with advancing age The distribution of the Sertoli cell index in patients less than 70 years of age with benign nodular hyperplasia only resembled that in patients in whom histology of the prostate was normal (Figs 4b and 4d) The correlation between the Sertoli cell index and age was negative in all groups, but the correlation was statistically significant only in the groups with BNH alone ($r = -0\ 344$ $p<0\ 01$) and BNH together with AGP ($r = -0\ 514$, $p = 0\ 05$)

The regression lines for the relation between the Sertoli cell index and age in men aged 50 years or more within various groups

TABLE 4 *Mean Sertoli Cell Index by Histology of the Prostate* and Age*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	(947 0)	–	563 0	–	–	–	–
50–59	633 9	816 7	810 1	1119 0	–	1063 3	–
60–69	737 4	(911 0)	751 6	780 6	512 0	672 0	(779 0)
70–79	–	(531 0)	628 8	806 1	–	601 3	–
80 +	–	–	351 6	645 2	–	611 0	–
All	688 5	778 4	662 3	777 7	512 0	766 7	–
S D	272 3	424 4	349 0	438 2	205.1	338 6	–

* For abbreviations and number of patients see Table 1
Figures in brackets refer to single observations
S D Standard deviation

of prostatic histology (N, BNH BNH+C, BNH+AGP) are presented in Figure 5 As carcinoma or atypical glandular proliferation of the prostate was not observed in patients under 50 years of age, and as cases in this age group were few, the age group 40 49 years was not included in the analysis Neither were the few cases with DA C alone or AGP alone suited for separate analyses The slopes of individual regression lines for the BNH BNH+C and the BNH+AGP groups were all negative and did not differ significantly from each other Men with normal prostatic histology were all under 70 years of age and revealed practically no alteration in the Sertoli cell index with advancing age

The Sertoli cell index in patients who had undergone transurethral resection or open prostatectomy for benign hyperplasia of the prostate appears from Table 5 The age specific means were similar to those of non operated patients (Table 2)

Three of the 4 patients who had been treated with oestrogenic hormones had Sertoli cell indices at zero (Table 6) One patient who had received oestrogens for only one month also had relatively few Sertoli cells As estimated from the testis weight in these patients, they all had considerable testicular atrophy

As a preliminary orientation prior to the multiple regression analysis, the relationship between the Sertoli cell index and the individual regressors was studied at simple correlation analysis (Table 7) Among the con-

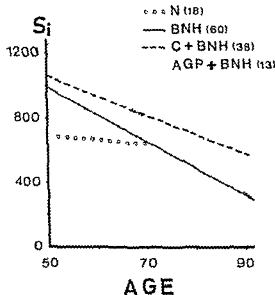


Fig 5 Regression of Sertoli cell index (S_i) on age in men aged 50 years or more with benign nodular hyperplasia only (BNH) benign nodular hyperplasia and carcinoma (C + BNH) benign nodular hyperplasia and atypical glandular proliferation (AGP + BNH) and normal histology (N) of the prostate Figures in brackets indicate number of cases in each group

TABLE 5 *Sertoli Cell Index in Patients Previously Subjected to Prostatic Surgery**

Age	No patients	Sertoli cell index		
		Mean	S D	Range
50–59	3	926 3	391 3	518–1298
60–69	3	737 3	575 3	222–1358
70–79	4	616 8	241 7	337– 873
80 +	7	416 9	239 4	95– 716
All	17	610 4	362 1	95–1358

* One patient subjected to both prostatectomy and treatment with oestrogenic hormones not included
S D Standard deviation

TABLE 6 *Sertoli Cell Index and Testis Weight (Grams), Age (Years) and Duration of Treatment in Patients who Received Oestrogenic Hormones (Diethylstilboestrol)*

Age	Duration of oestrogen treatment	Sertoli cell index	Testis weight*
69	1 month	252	15 5
66	12 "	0	1 9
76	4 years	0	14 9
79	4 "	0	10 7

* For details, see *Harbitz* (1973a)

tinuous variables, only age was significantly correlated with the Sertoli cell index Among bivariate variables, treatment with steroid hormones other than oestrogens and death from malignant tumour showed a positive and significant correlation with the Sertoli cell index None of the groups of prostatic histology *per se* or other variables, either continuous or bivariate demonstrated an association with the Sertoli cell index at the chosen level of significance

Multiple Regression Analysis

At the stepwise regression analysis, treatment with steroid hormones other than oestrogens (X_{10}) caused the greatest reduction in the variance of the Sertoli cell index and was selected prior to age (X_{13}) and benign nodular hyperplasia of the prostate (X.) The Sertoli cell index was negatively correlated with age ($p=0001$) whereas the associations to steroid hormone treatment and BNH were positive ($p=0002$ and $p\approx0033$ respectively) These regressors were the only three to be selected at the chosen significance level Their multiple correlation coefficient (R) was low (0 373), and this set of regressors explained only 13 9 per cent (R^2) of the individual variation in the Sertoli cell index

At full regression analysis including the regressors for prostatic histology (X -X_5), the correlations between the Sertoli cell index and age and steroid hormone treatment were confirmed (Table 8) Practically no alterations of the correspondent partial correlation coefficients and their levels of significance were caused by the introduction of prostatic histology in the regression The application of the linear regression equation for the predicted value of the Sertoli cell index (X_1) gives that steroid hormone treatment (X_{10}) in this series on the average would increase

TABLE 7 *Relationship between Sertoli Cell Index and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variable	X_1 Sertoli cell index ($n_1 = 141$)	
	Correlation coefficient	Significant at level
*Histology of the prostate**		
X_2 BNH (n = 113)	0 027	0 754
X_3 AGP (n = 14)	0 054	0 523
X_4 C (n = 41)	0 088	0 298
X_5 DA (n = 5)	0 037	0 663
Cause of death		
X_6 Cardiovascular disease (n = 78)	–0 095	0 262
X_7 Malignant tumour (n = 30)	0 178	0 034
Duration of final illness		
X_8 1–7 days (n = 32)	0 020	0 815
X_9 >7 days (n = 73)	0 029	0 737
Other		
X_{10} Steroid hormone treatment† (n = 16)	0 238	0 004
X_{11} Diabetes mellitus (n = 6)	0 066	0 435
X_{12} Liver cirrhosis (n = 4)	–0 079	0 351
X_{13} Age (n = 141)	0 232	0 006
X_{14} Body weight (n = 141)	0 031	0 715
X_{15} Body length (n = 141)	0 143	0 092

* For abbreviations see Table 1

n_1 Number of cases in which Sertoli cell index was recorded

n Number of cases in which the characteristic in question was present (for bivariate variables) or recorded (for continuous variables)

† Other than oestrogenic hormones

TABLE 8 *Sertoli Cell Index and Histology of the Prostate Full Regression Analysis*

Explanatory variable	X_1 Sertoli cell index ($n_1 = 141$)		
	Partial correlation coefficient	Partial regression coefficient	Significant at level
*Histology of the prostate**			
X_2 BNH (n = 113)	0 161	163 8	0 061
X_3 AGP (n = 14)	0 094	108 8	0 277
X_4 C (n = 41)	0 169	135 7	0 050
X_5 DA (n = 5)	0 043	84 4	0 621
Other			
X_{13} Age (n = 141)	–0 318	–12 9	<0 001
X_{10} Steroid hormone treatment† (n = 16)	0 255	282 5	0 003
Multiple correlation coefficient (R)	0 409		<0 001

* For abbreviations see Table 1

† Other than oestrogenic hormones.

n and n_1 For explanation see Table 7

the Sertoli cell index by about 280, whereas increasing age resulted in a reduction in the Sertoli cell index at a rate of about 13 per year

A significant correlation between the Sertoli cell index and prostatic carcinoma *per se* (X_4) appeared at full regression analysis, but the partial correlation coefficient and its level of significance were low The level of significance for the correlation between the Sertoli cell index and BNH (X_2) observed at stepwise regression analysis was reduced to 6 1 per cent The inclusion of all histological groups in the analysis did not increase the explanatory value of the regression substantially ($R^2 = 0.167$) Neither was the difference between individual regression coefficients for the groups of prostatic histology statistically significant ($F = 2.292$, $f_1 = 4$ $f_2 = 134$ $0.05 < p < 0.10$)

COMMENT

Quantitative studies of Sertoli cells in the human testis are few and only relative measures have been applied (*Roosen Runge* 1956, *Mancini et al* 1960) In the present investigation an index for the absolute number of Sertoli cells based upon the count of Sertoli cell nuclei was determined and the results cannot be compared directly with those in previous reports In agreement with previous experience (*Tillinger* 1957 *Mancini et al* 1960) Sertoli cells were easily distinguished from the germinative epithelium and the reproducibility for the determination of the Sertoli cell number was within acceptable limits.

It was demonstrated that the number of Sertoli cells decreased significantly with age Patients with benign prostatic hyperplasia revealed declining Sertoli cell index with increasing age whether the hyperplasia occurred alone or together with carcinoma or atypical glandular proliferations Individual observations varied over a wide range in all age groups and Sertoli cell numbers in patients in the eighth and ninth decades were sometimes within the range of observations applying to the younger age groups This complicates the evaluation based on the Sertoli cell number, of the endocrine environment in elderly men However, the declining number of Sertoli cells may provide morphological evidence for an altered endocrine state, which in its turn may be of importance in the pathogenesis of prostatic neoplasia and hyperplasia

The relationship between prostatic histology and the number of Sertoli cells *per se* was analysed at full multiple regression analysis This correlation was not clearly significant for any of the histological groups and the difference between the partial regression coefficients was not statistically significant This means that the Sertoli cell number by itself is of no help in the diagnostic discrimination between various histological patterns in the prostate

Some patients had been given steroid hormones other than oestrogens for various periods before death Their mean Sertoli cell index was considerably higher than that applying to the whole series and Sertoli cell index (X_1) showed a positive and statistically significant correlation to treatment with steroid hormones (X_{10}) at multiple regression analysis It cannot be decided on the basis of the observations in this series however, whether the apparent proliferation of Sertoli cells resulted from a direct effect of steroid hormones or through more complex mechanisms

It appears from the observations in the present study that prostatic surgery for benign hyperplasia does not affect the number of Sertoli cells This may have functional implications for the germinative epithelium in these patients since androgens elaborated by the Sertoli cells are probably responsible for the normal maintenance of spermatogenesis (*Lacy* & *Pettitt* 1970)

Oestrogenic steroids in high dosage depress pituitary gonadotrophin (FSH) production which controls tubular function resulting in regression of spermatogenic epithelium The Sertoli cells however are invariably the last to disappear in progressive degeneration of the tubules (*Johnsen* 1967 Nevertheless among the few patients in the present series

who had received oestrogenic hormones, the ones treated for 12 months or more presented complete loss of Sertoli cells

The mean Sertoli cell index in the four patients with liver cirrhosis was markedly lower than the mean applying to the whole series This may be related to pituitary suppression and hypogonadotrophic hypogonadism secondary to the high levels of circulating endogenous oestrogen observed in male cirrhotics (*Coppage* & *Cooner* 1965, *Lederer* & *Bataille* 1967)

In conclusion, the total number of Sertoli cells declines with advancing years and is not significantly related to the histology of the prostate as such The wide variation in individual observations and the possibility that Sertoli cells are involved in the production of both androgenic and oestrogenic hormones makes it difficult to evaluate the hormonal state of elderly men on the basis of their Sertoli cell numbers The present study revealed no distinct differences in the relationship between the Sertoli cell number and age whether the patients had benign nodular hyperplasia alone or it was associated with carcinoma or atypical glandular proliferations of the prostate

---

I am indebted to Professor *Helge Stalsberg* M D Institute of Medical Biology University of Tromsø Norway for reading the manuscript. Actuary *Ingar Holme* Cand real The Norwegian Computing Center Oslo Norway performed the computer analysis

The technical assistance of Mrs *B Eriksen* and Mrs *E Jaeg* is gratefully acknowledged

Part of this study was performed while the author was a research fellow under *The Norwegian Society for Fighting Cancer* and *The University of Oslo* Financial support was thankfully received from *Fru dr Ludvig Larsens legat til kreftens bekjempelse Grosserer V A Stangs Legat til Kreftsykdommers Bekjempelse* and *Doktor Alexander Malthes Legat*

REFERENCES

1 *Armitage P* Statistical methods in medical research Blackwell Scientific Publications, Oxford and Edinburgh 1971 504 pp

2 *Bell J B G Vinson G P & Lacy, D* Studies on the structure and function of the mammalian testis. III *In vitro* steroidogenesis by the seminiferous tubules of rat testis. Proc roy Soc B. *176* 433–443 1971

3 *Berthrong, M, Goodwin, W E & Scott, W W* Estrogen production by the testis J clin Endocr 9 579 592, 1949

4 *Bloom W & Fawcett D W* A textbook of histology 9 ed Saunders Company, Philadelphia, London, Toronto 1968 p 687–689

5 *Coppage, W S Jr & Cooner, A E* Testosterone in human plasma. New Engl J Med *273* 902 907, 1965

6 *Geissendorffer, R* Hat die These von der hormonalen Genese der sogenannten Prostatahypertrophie heute noch Geltung? Helv chir Acta *27* 273–291 1960

7 *Hanafy H M, Gursel, E & Veenema, R J* A possible role of Sertoli cells in prostatic cancer refractory to estrogen A preliminary report J Urol *108* 914–917, 1972

8 *Harbitz T B* Testis weight and the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *81* 148–158 1973a.

9 *Harbitz, T B* Morphometric studies of the Leydig cells in elderly men with special reference to the histology of the prostate An analysis in an autopsy series Acta path microbiol scand Section A. *81* 301–314, 1973b

10 *Harbitz T B & Haugen O A* Histology of the prostate in elderly men A study in an autopsy series Acta path microbiol scand Section A *80* 756–768 1972

11 *Haugen O A & Harbitz T B* Prostatic weight in elderly men An analysis in an autopsy series Acta path. microbiol scand Section A *80* 769 777 1972

12 *Hooker C W* The intertubular tissue of the testis In Johnson A D Gomes W R. & Vandemark N L (Eds) The Testis vol I Academic Press New York and London 1970 p 483 550

13 *Huggins C & Hodges C V* Studies on prostatic cancer I The effect of castration of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate Cancer Res *1* 293–297 1941

14 *Huggins C Scott W W & Hodges C V* Studies on prostatic cancer III The effects of fever of desoxycorticosterone and of estrogen on clinical patients with metastatic carcinoma of the prostate J Urol *46* 997 1006 1941a.

15 *Huggins C Stevens R E Jr & Hodges C V* Studies on prostatic cancer II The effects of castration on advanced carcinoma of the prostate gland Arch. Surg *43* 209–223 1941b

16 *Isurugi K* Plasma hormones and prostatic

dysfunction In Griffiths, K. & Pierrepoint, C G (Eds) Some Aspects of the Aetiology & Biochemistry of Prostatic Cancer Third Tenovus Workshop Tenovus Workshop Publications, Cardiff 1970 p 33-38

17 *Johnsen, S G* The mechanisms involved in testicular degeneration in man Acta endocr (Kbh) Suppl *124* 17-40, 1967

18 *Johnsen, S G* Investigations into the feed back mechanism between spermatogenesis and gonadotropin level in man In Rosemberg, E & Paulsen, C A (Eds) The Human Testis Advances in Experimental Medicine and Biology, vol 10 Plenum Press, New York and London 1970 p 231-248

18 *Lacy, D & Pettitt, A J* Sites of hormone production in the mammalian testis, and their significance in the control of male fertility Brit. med Bull *26* 87-91, 1970

19 *Lederer, J & Bataille, J P* Mecanisme de l hypogonadisme masculin au cours de la cirrhose Ann Endocr *28* 821-826, 1967

20 *Lynch, K M, Jr & Scott, W W* The lipid content of the Leydig cell and Sertoli cell in the human testis as related to age, benign prostatic hyperplasia, and prostatic cancer J Urol *64* 767-776, 1970

21 *Mancini, R E, Narbaitz, R & Lavieri, J C* Origin and development of the germinative epithelium and Sertoli cells in the human testis Cytological, cytochemical, and quantitative study Anat. Rec *136* 477-483 1960

22 *Marmorston, J* Urinary hormone metabolite levels in patients with cancer of the breast, prostate and lung Ann N Y Acad Sci *125* 959-973 1966

23 *Moore R A* Male secondary sexual organs In Lansing, I I (Ed) Cowdry s Problems of Ageing Biological and Medical Aspects 3 ed Williams & Wilkins Company, Baltimore 1952 p 686-707

24 *Robson, M C* Cirrhosis and prostatic neoplasms Geriatrics *21* 150-154, 1966 (Dec.)

25 *Roosen Runge, E C* Quantitative investigations on human testicular biopsies I Normal testis Fertil and Steril *7* 251-261, 1956

26 *Scheffé, H* The analysis of variance John Wiley & Sons, Inc, New York 1959 p 25-51

27 *Snedecor, G W & Cochran, W G* Statistical methods 6 ed The Iowa State University Press, Ames, Iowa 1967 593 pp

28 *Sohval, A R* The anatomy and endocrine physiology of the male reproductive system In Velardo J T (Ed) The Endocrinology of Reproduction Oxford University Press 1958 p 243-312

29 *Sommers, S C* Endocrine changes with prostatic carcinoma. Cancer *10* 345-358, 1957

30 *Stern, E, Hopkins, C E, Weiner, J M & Marmorston, J* Hormone excretion patterns in breast and prostate cancer are abnormal Science *145* 716 719, 1964

31 *Teilum, G* Oestrogen production by Sertoli cells in the etiology of benign senile hypertrophy of the human prostate Testicular lipoid cell ratio' and oestrogen androgen quotient in human male Acta endocr (Kbh) *4* 43-62 1950

32 *Tillinger, K G* Testicular morphology A histo pathological study with special reference to biopsy findings in hypogonadism with mainly endocrine disorders and in gynecomastia Acta endocr (Kbh) Suppl *30* 21, 1957

Acta path microbiol scand Section A *81, 5,* 715 724, 1973

SCHEDULE DEPENDENCY OF THE ANTILEUKEMIC ACTIVITY OF THE *PODOPHYLLOTOXIN-DERIVATIVE* VP 16-213 (NSC-141540) *IN L1210* LEUKEMIA

PER DOMBERNOWSKY and NIS I NISSEN

Finsen Institute, Department of Internal Medicine, 2100 DK Copenhagen, Denmark

The schedule dependency of the antileukemic activity and toxicity of the podophyllotoxin derivative VP 16 213 (NSC 141540) was investigated against the L1210 ascites tumour in N/D mice In the toxicity experiments LD10 for intraperitoneal (i p) treatment on day 1 only, once daily for five days and once daily day 1, 5, 9 and 13 was found to be 47 0, 7 7 and 33 6 mg/kg, respectively In the therapy experiments treatment was started 24 hours after inoculation of 10^5 L1210 cells i p The optimal concentrations gave for treatment on day 1 only 75 per cent increase in lifespan and 13 per cent cures Daily treatment for 5 days was superior to treatment day 1 only, this was again exceeded by treatment at 2-4 days intervals Even better results were obtained with treatment at 4 days intervals (cure rate 63 per cent), while treatment at 6 8 days intervals gave inferior results Divided treatment every 3 hours for 24 hours demonstrated a significant superiority over a single treatment. This together with the mechanism of action could indicate, that VP 16-213 is a cell cycle specific drug

The cytostatic activity of derivatives of the plants podophyllum peltatum and podophyllum emodi has been known for many years (9, 11) but active compounds for systemic use have only been made available recently (10) Among a number of semisynthetic podophyllotoxin-derivatives, 4' demethyl-epipodophyllotoxin-9-(4,6-0-2-thenylidene-β-D-glucopyranoside) (NSC-122819) or VM 26 (Fig 1) was identified by its striking antitumour activity in the L1210 leukemia (21), and it has also been proved to be of some clinical use in the treatment of lymphomas and solid tumours (5 6, 13, 14, 24)

ed to P
ment of
2100 DK

Another semisynthetic derivative, 4'-demethyl-epipodophyllotoxin-9 (4,6 0-ethylidene-β D glucopyranoside) (NSC-141540) or VP 16-213 (Fig 1), chemically closely related to VM 26, has in preliminary screening shown even better therapeutic results (23) and following a phase I clinical trial (15) it is at present undergoing a more extensive clinical trial in Acute Leukemia Group B and other groups

For both drugs, *Stähelin's* preliminary reports suggested that intermittent treatment was superior to daily treatment in the mouse tumours (21, 23) As the proper scheduling of cytostatic agents can be of decisive importance for the therapeutic results and as the L1210 leukemia has been shown to be predictive to the clinical situation (7, 25), we undertook a comparative study of various

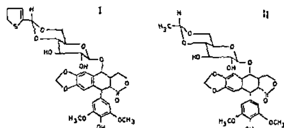


Fig 1 Chemical structure of

I 4' demethyl-epipodophyllotoxin 9-(4,6-0-2-thenylidene-β D glucopyranoside) (NSC-122819), VM 26

II 4'-demethyl-epipodophyllotoxin-(4, 6 0-ethylidene-β-D-glucopyranoside) (NSC-141540), VP 16-213

treatment schedules of VP 16-213 in the L1210 leukemia in mice

MATERIALS AND METHODS

Mice and Tumour

First generation male hybrids of female random-bred Swiss mice and male inbred DBA/2 mice (N/D mice) were used in all experiments All mice used in each experiment were born within one week and weighed 18 22 gm

The strain of leukemia L1210 used was obtained from Southern Research Institute, Birmingham, Alabama in 1969 and has since been maintained in DBA/2 mice in our laboratory by weekly intraperitoneal (i p) inoculation of 10^5 cells

Tumour Transplantation

Ascites tumour cells from one or more animals inoculated 6-7 days earlier were removed under sterile conditions, pooled, and kept below 4° C The number of cells was counted in a hemocytometer, and the suspension was diluted with isotonic NaCl until 10^5 tumour cells were contained in 0 2 ml, which was then given i p to each test mouse Appropriate controls with respect to sterility of the tumour (bacteriological examinations), homogeneity (differential counts) and viability (nigrosin test) were carried out in each experiment (3).

Drug and Drug Administration

VP 16 213 was supplied from Sandoz Ltd, Basle, Switzerland in vials containing 100 mg drug in a solvent described below. The solubility of the drug in water is poor and a solvent composed of Tween 80 20 mg, Polyethylene glycol 300 600 mg, Benzyl alcohol 30 mg, Citric acid (anhydrous) 2 mg and Absolute alcohol ad 1 ml was used for each 20 mg VP 16-213 The drug was prepared just before administration and diluted with isotonic NaCl until the dose per gram of mice was contained in 0 01 ml The mice were weighed before each injection and the appropriate dose was administered i p

Toxicity Experiments

Mice were randomized in groups of 10 and each group was given the amount of drug as in schedules described under results The toxic effect of each dose and schedule was measured by the per cent of mice dying within 30 days after the last injection 5 8 groups of mice were treated with doses which caused a lethality ranging from 0 to 100 per cent The lethal dose for 10 per cent of hybrid mice (LD10) and 50 per cent (LD50), with confidence limits, was calculated by a computerized program of logit analysis (2)

Therapy Experiments

After transplantation, mice were randomized into a control group of 18-24 mice, and various treat

three times, and the data pooled Antitumour effect of the drug was evaluated by comparing prolongation of median survival time (MST) in tumour bearing treated mice and in untreated tumour-bearing controls, and per cent increase in life span (per cent ILS) was calculated (16) When the

TABLE 1 *LD10 and LD50 Dose of VP 16-213 per Treatment Day in N/D Mice*

Schedule	*LD10*	*LD50*
day 1 only	47 0 mg/kg (36 5–60 5)	78 0 mg/kg (66 8–91 0)
once daily for five days	7.7 mg/kg (5 9– 8 9)	10 8 mg/kg (9 7–12 1)
once daily day 1, 5, 9 and 13	33 6 mg/kg (28 2–40 0)	43 9 mg/kg (39 2–49 1)

Numbers in brackets indicate 95 per cent confidence limits

TABLE 2 *Effect of VP 16 213 in Various Schedules and Doses on Survival Time of N/D Mice Transplanted with L1210 Leukemia*

VP 16 213 treatment schedule	mg/kg per treatment day	median survival time in days (MST)	per cent increase in lifespan over controls (ILS)	fraction of "cures"	per cent "cures"
once day 1 only	120	5.0	0	0/24	0
–	90	5.0	0	0/24	0
–	60	14.0	75	3/24	13
–	45	14.0	75	3/24	13
–	30	14.0	75	2/24	8
–	15	13.5	69	0/24	0
–	7.5	12.0	50	0/24	0
once daily for five days	20	7.0	0	0/24	0
–	15	8.0	0	0/24	0
–	10	16.0	100	5/24	21
–	7.5	19.0	138	0/24	0
–	5.0	18.0	125	0/24	0
–	2.5	14.0	75	0/24	0
–	1.3	13.0	63	0/24	0
–	0.6	10.0	25	0/24	0
once daily for nine days	10	8.5	6	1/24	4
–	7.5	29.0	263	9/24	38
–	5.0	20.0	150	3/24	13
–	3.8	17.5	119	0/24	0
–	2.5	15.5	94	0/24	0
once daily day 1, 3 and 5	30	8.0	0	3/16	19
–	20	28.0	250	5/16	31
–	15	30.0	275	6/16	38
–	10	23.5	194	3/16	19
–	5	15.0	88	0/16	0
once daily day 1 3, 5 and 7	30	8.0	0	0/16	0
–	20	16.5	106	3/16	19
–	15	29.0	263	3/16	19
–	10	23.5	194	1/16	6
–	5	18.0	125	0/16	0
once daily day 1 3 and 7	50	6.5	0	0/16	0
–	37.5	7.0	0	1/16	6
–	25	25.5	219	5/16	31
–	12.5	28.0	250	3/16	19
–	6.3	16.5	106	1/16	6
once daily day 1 and 5	50	20.5	156	9/24	38
–	40	>60.0	>650	12/24	50
	30	>60.0	>650	14/24	58
–	20	20.0	150	5/24	21
–	10	18.0	125	1/24	4

VP 16 213 treatment schedule	mg/kg per treatment day	median survival time in days (MST)	per cent increase in lifespan over controls (ILS)	fraction of "cures"	per cent "cures"
once daily day 1, 5 and 9	50	18.0	125	6/24	25
–	40	>60.0	>650	15/24	63
–	30	>60.0	>650	14/24	58
–	20	34.0	325	8/24	33
–	10	17.5	119	0/24	0
once daily day 1, 5, 9 and 13	50	13.0	63	0/24	0
–	40	19.0	138	3/24	13
–	30	>60.0	>650	12/24	50
–	20	>60.0	>650	13/24	54
–	10	17.5	119	1/24	4
once daily day 1, 5, 9, 13, 17 and 21	50	7.5	0	0/24	0
–	40	>60.0	>650	12/24	50
–	30	>60.0	>650	12/24	50
–	20	29.0	263	9/24	38
–	10	30.5	281	6/24	25
–	5	19.0	138	0/24	0
once daily day 1 and 7	50	18.0	125	4/16	25
–	40	42.0	425	7/16	44
–	30	26.5	231	0/16	0
–	20	18.0	125	0/16	0
–	10	16.0	100	1/16	6
once daily day 1, 8, 15 and 22	60	13.5	69	0/24	0
–	45	25.5	219	2/24	8
–	30	37.5	369	5/24	21
–	15	19.5	144	2/24	8
–	7.5	13.5	69	0/24	0
–	3.8	12.5	56	0/24	0
once daily day 1 and 9	50	30.5	281	9/24	38
–	40	31.0	288	10/24	42
–	30	38.0	375	8/24	33
–	20	21.5	169	0/24	0
–	10	14.5	81	0/24	0
every 3 h for 24 hours day 1 only	90	6.0	0	0/16	0
–	60	5.0	0	0/16	0
–	45	>60.0	>650	8/16	50
–	30	31.0	288	6/16	38
–	15	16.5	106	0/16	0
–	7.5	13.0	63	0/16	0
–	3.8	12.0	50	0/16	0

VP 16 213 treatment schedule	mg/kg per treatment day	median survival time in days (MST)	per cent increase in lifespan over controls (ILS)	fraction of 'cures'	per cent "cures"
every 3 h for 24 hours day 1 and 5	60	5 0	0	0/16	0
–	45	5 0	0	4/16	25
–	30	>60 0	>650	16/16	100
–	15	23 5	194	4/16	25
–	7 5	17 0	113	0/16	0
–	3 8	14 5	81	0/16	0
every 3 h for 24 hours day 1, 5 and 9	60	6 0	0	0/16	0
–	45	9 0	13	2/16	13
–	30	>60 0	>650	9/16	56
–	15	35 5	344	6/16	38
–	7 5	23 5	194	0/16	0
–	3 8	16 0	100	0/16	0

The data were pooled from 2 or 3 experiments on each concentration and schedule
Median survival time of untreated control mice was 8 0 days
Cured mice were recorded as having lived 60 days

maximum MST was noted at more than one dosage level, selection of the optimal dose was based on the entire survival pattern and per cent of sixty day survivors Sixty day survivors from start of an experiment were defined as "cures", as we found that only 1 of 50 mice observed for 100 days died after day 60 with persistance of tumour In no instance did an untreated control animal survive

The Wilcoxon one tailed test for two samples was used for the statistical evaluation.

RESULTS

Toxicity Experiments

Were performed on day 1 only, once daily for 5 days, and once daily day 1, 5, 9 and 13 with doses per injection ranging from 6 to 180 mg/kg

The mice died from 1 to 13 days after the last administration of VP 16-213 LD10 and LD50 with 95 per cent confidence limits in mg/kg per treatment day are shown in Table 1 The tolerated dose was higher for the intermittent schedule than for daily treatment for 5 days both with respect to individual and total doses

In experiments using 150 mg/kg VP 16-213 on day 1 and 50 mg/kg once daily for 5 days, control experiments were included where the corresponding VP 16-213 solvent was given alone No deaths were observed from the solvent, while all mice died from the VP 16-213 doses mentioned

Therapy Experiments

Are summarized in Table 2 and in Fig 2 and 3 In all experiments the median survival time of the untreated control mice was 8 0 days

Daily Treatment

Treatment on day 1 only gave a moderate increase in MST to 14 0 days, and 13 per cent 60 day survivors ("cures") with the optimal concentration Slightly longer ($p<0 05$) MST was found when treatment was given for 5 days and 21 per cent were "cures" Still better results ($p<0 005$ and $p<0 01$, respectively) were obtained with treatment for 9 days when MST was 29 0 days and 9/24 mice survived

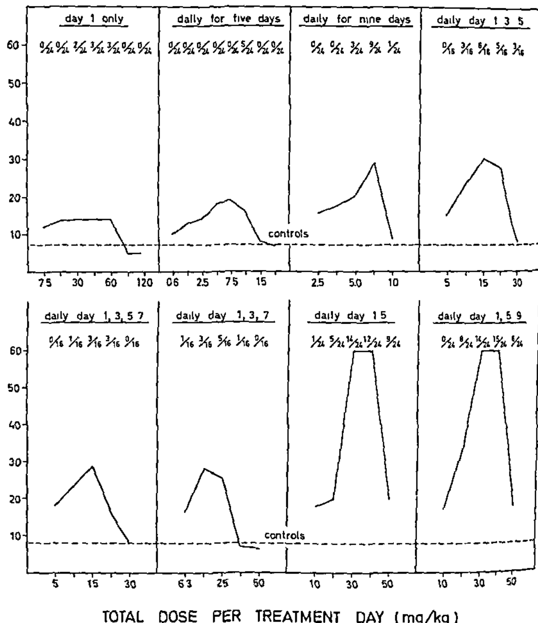


Fig 2

Fig 2 and 3 Comparison of the effectiveness of different VP 16 213 treatment schedules against early L1210 ascites tumor The inoculum was 10^5 L1210 cells i p and treatment was started 24 hr later The numbers in brackets indicate fraction of sixty day survivors

Treatment at 2-4 Days Intervals

Treatment day 1, 3 and 5, day 1, 3, 5 and 7 and day 1, 3 and 7 showed significant increase in lifespan compared with treatment day 1 only ($p<0.05$) with MST of 28 0 to 30 0 days and a cure rate from 19 to 38 per cent.

Treatment at 4 Days Intervals

Intermittent treatment every fifth day proved significantly superior ($p<0.025$ to 0 005) to treatment day 1 only, treatment once daily for 5 and 9 days and to treatment at 2-4 days intervals With treatment day 1 and 5 or 1, 5, and 9 or 1, 5, 9, and 13 or 1, 5, 9,

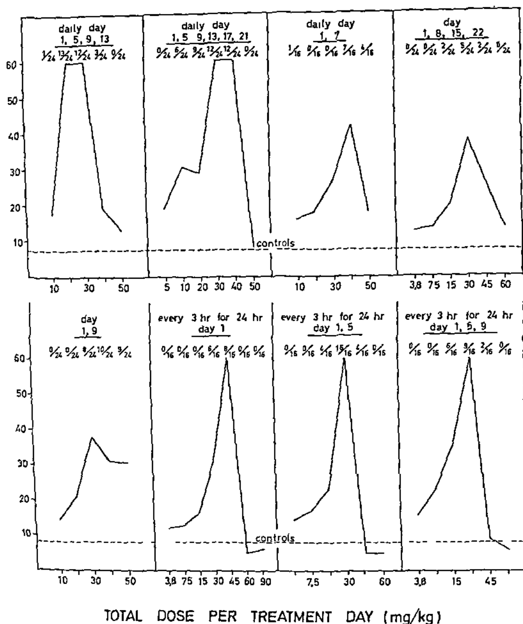


Fig 3

13, 17, and 21 the optimal dose was 20 to 40 mg/kg, MST more than 60 days and the "cure" rate from 50 to 63 per cent The two more prolonged intermittent schedules were not significantly ($p > 0\ 10$) better than the short intermittent schedules day 1 and 5 or 1, 5, and 9

Treatment at 6-8 Days Intervals

When the intervals between the injections were prolonged to 6-8 days (treatment day 1 and 7, treatment day 1, 8, 15, and 22 and treatment day 1 and 9) the MST and cure rate seemed to decrease again (MST 37 5 to 42 0 days, cure rate 21 to 42 per cent.

Divided Treatment

If treatment was given on day 1 only, the divided treatment every 3 hours for 24 hours showed a striking superiority ($p<0.01$) over a single injection (MST>60 days versus 14 days, and 50 per cent cures versus 13 per cent cures).

The same divided schedule applied on day 1 and 5 gave significant better results ($p<0.025$) compared with a single injection day 1 and 5. This schedule was also significant better than all other schedules ($p<0.05$ to 0.005), while MST and cure rate with divided treatment on day 1, 5, and 9 was in the same range as standard treatment day 1, 5 and 9 ($p>0.10$).

DISCUSSION

The podophyllotoxin derivative VP 16 213 was found to posess a very high antitumour activity against the L1210 ascites tumour with divided treatment or treatment once daily every fourth day being the best schedules.

Since 1955 L1210 leukemia has been used in the primary drug screen in the Cancer Chemotherapy National Service Center program (12). In the early period with drugs such as methotrexate it was only possible to obtain a moderate increase in life span and no cures (8). Following the introduction of cyclophosphamide, the nitrosoureas and cytosine arabinoside the treatment became more effective and in several experiments a large increase in life span of the mice and a high per cent of cures could be demonstrated with the best schedules (18, 19, 20). Our results and those of *Stahelin* (23) have shown an extremely high activity of VP 16 213 against the L1210 ascites tumour with up to 100 per cent long term survivors on the optimal schedules, thus placing VP 16 213 as one of the most active drugs against this tumour.

Demonstration of a pronounced antitumour effect in the L1210 system is important as it has been shown retrospectively that regardless of the basis for their initial selection and the human cancer which they were effective against most clinically active drugs have a very high anti L1210 activity (7).

The principal aim of examining different treatment schedules in the L1210 tumour system is to obtain information on the optimal conditions of drug use for later use in the clinical treatment. The most effective treatment schedule is the schedule which exerts maximum selective kill of the tumour cells relative to normal sensitive cells. This together with the differential rate of recovery of the most sensitive normal cells relative to the rate of recovery of the tumour cell population determines the outcome of the therapy (17).

The single treatment on day 1 only usually gives a baseline activity level for alternative schedules, and provides an estimate of the percentage of leukemic cells killed by a given drug dose (18). With *Skipper's* method a calculated tumour cell kill of 99.998 per cent (1 of 50000 cells survives) was obtained in our experiments after one injection of 45 mg/kg VP 16 213.

The superior results obtained with an interval of four days between the injections are compatible with a higher per cent kill of the tumour cells and enough time for recovery of the normal cells. Treatment with shorter intervals led to inferior results probably due to decreased tolerance of the normal host cells. Treatment with longer intervals up to 7 to 9 days also gave inferior results, but in this case probably due to tumour cell recovery between the injections.

Quite different from these spaced treatments is the continuous treatment or its practical analogue, treatment every 3 hours (usually called divided treatment). With this schedule most of a metabolically random L1210 population is exposed to the drug one or more times during the sensitive phase of the cell cycle (4, 20). Such a divided treatment on day 1 only gave in our experiments a dramatic increase in MST and per cent cures. Comparison between divided and intermittent schedules day 1 and 5 also demonstrated the superiority of the divided treat

ment, while no such difference is found when treatment is given day 1, 5 and 9, probably due to increase in toxicity

The mechanism of action of VP 16 213 is not known in detail but as with the other podophyllotoxin derivative VM 26, mitotic arrest in the metaphase is first seen, and after 30 60 minutes the entry of cells into the mito tic phase is blocked (22 23) This together with the superiority of divided treatment day 1 and 1 and 5 compared with one single in jection on the same days, indicates that VP 16 213 has its main action on proliferating cells (17)

This study shows the decisive importance of proper scheduling for the outcome of the rapy This was demonstrated as early as 1956 in Goldin s now classic experiments where intermittent treatment with methotrexate gave superior results against L1210 leukemia when compared with daily treatment (8) The findings became especially of great clini cal relevance, when a subsequent controlled study by Acute Leukemia Group B confirmed the superiority of the intermittent methotrex ate schedule in lymphoblastic leukemia in children (1)

Whether the superiority of intermittent schedule of VP 16 213 in our animal experi ments also has a clinical bearing is too early to say At this time a phase I study in humans has demonstrated some antitumour effect in both lymphomas and solid tumours (15) However, for further evaluation of the drug clinically, phase II studies and investigations of VP 16 213 in combination with other drugs have to be performed Such studies are now in progress in several centers

---

The authors are indebted to Miss *J Andersen* Mrs. *N Arnkær* and Mr *J Dusing* for technical assistance and to Mr *S Olesen Larsen* for statisti cal evaluation.

This work was supported by grants from the *Daell Foundation The Danish Cancer Society The Danish Hospital Foundation for Medical Research Reg on of Copenhagen* and the *Hasselbalch Found at on*

46*

REFERENCES

1 *Acute Leukemia Group B* New treatment schedule with improved survival in childhood leukemia J Amer Med Ass *194* 187–193, 1965
2 *Armitage, P* Statistical methods in medical research Blackwell Scientific Publications Ox ford and Edinburgh 1971, p 359
3 *Dombernowsky, P* The proliferation kinetics of L 1210 ascites tumour Acta path micro biol. scand Section A 80 603–611, 1972
4 *Dombernowsky P & Hartmann N R* Ana lysis of variations in the cell population kine tics with tumor age in the L1210 ascites tu mor Cancer Res *32* 2452–2458 1972
5 *Dombernowsky, P, Nissen, N I & Larsen, V* Clinical investigation of a new podophyllum derivative epipodophyllotoxin, 4 demethyl 9 (4 6 0 2 thenylidene β D glucopyranoside) (NSC 122819) in patients with malignant lymphomas and solid tumors Cancer Chem other Rep 56 71–82, 1972
6 *European Organization for Research on the Treatment of Cancer Co operative Group for Leukaemias and Haematosarcomas* Clinical screening of epipodophyllotoxin VM26 in ma lignant lymphomas and solid tumours Brit Med J 2 744–748 1972
7 *Goldin A Serpick A A & Mantel, N* Ex perimental screening procedures and clinical predictability value Cancer Chemother Rep *50* 173 218 1966
8 *Goldin A Venditti J M Humphreys S R & Mantel N* Modification of treatment sche dules in the management of advanced mouse leukemia with amethopterin J Nat Cancer Inst *17* 203 212 1956
9 *Kaplan I W* Condylomata acuminata New Orleans Med Surg J 94 388–390 1942
10 *Keller Juslen C Kuhn M Wartburg A von & Stahelin H* Synthesis and antimitotic ac tivity of glycosidic lignan derivatives related to podophyllotoxin J Med Chem *14* 936–940 1971
11 *Kelly M G & Hartwell J L* The biological effects and the chemical composition of podo phyllin. A Review J Nat. Cancer Inst *14* 967–1010 1954
12 *Leiter J & Schneiderman M A* Screening data from the cancer chemotherapy national service center screening laboratories Cancer Res *19* 31 193 1959
13 *Muggia F M* Phase I study of 4 demethyl epipodophyllotoxin β D thenylidene glycoside (PTG NSC 122819) Proc Amer Ass Can cer Res *11* 58 1970
14 *Muggia F M Selawry O S & Hansen H H* Clinical studies with a new podophyllo toxin derivative epipodophyllotox n 4 deme

thyl 9 (4 6-0-2 thenylidene-β D glucopyranoside) (NSC 122819) Cancer Chemother Rep 55 575-581, 1971

15 *Nissen, N I, Larsen V, Pedersen, H & Thomsen, K* Phase I clinical trial of a new antitumor agent, 4 demethyl-epipodophyllotoxin 9 (4 6 0-ethylidene β D glucopyranoside) (NSC 141540, VP 16 213) Cancer Chemother Rep 56, 769-777, 1972

16 Protocols for screening chemical agents and natural products against animal tumors and other biological systems Cancer Chemother Rep 25 1-66, 1962

17 *Skipper, H E, Schabel, F M, Jr, Mellett, L B, Montgomery, J A, Wilkoff, L. J, Lloyd, H H & Brockman, R W* Implications of biochemical, cytokinetic, pharmacologic, and toxicologic relationship in the design of optimal therapeutic schedules. Cancer Chemother Rep 54 431-450, 1970

18 *Skipper, H E, Schabel, F M, Jr & Wilcox W S* Experimental evaluation of potential anticancer agents XIII On the criteria and kinetics associated with curability of experimental leukemia Cancer Chemother Rep 35 1-111 1964

19 *Skipper H E Schabel, F M Jr & Wilcox W S* Experimental evaluation of potential anticancer agents XIV Further study of certain basic concepts underlying chemotherapy of leukemia Cancer Chemother Rep 45 5-28 1965

20 *Skipper, H E, Schabel, F M, Jr & Wilcox W S* Experimental evaluation of potential anticancer agents. XXI Scheduling of arabinosylcytosine to take advantage of its S phase specificity against leukemia cells. Cancer Chemother Rep 51 125-165, 1967

21 *Stahelin H* 4' demethyl epipodophyllotoxin thenylidene glucoside (VM 26), a podophyllum compound with a new mechanism of action Europ J Cancer 6 303 311, 1970

22 *Stahelin H* Personal communication 1972.

23 *Stahelin, H* Activity of a new glycosidic lignan derivative (VP 16 213) related to podophyllotoxin in experimental tumors. Europ J Cancer 9 215 221, 1973

24 *Trempe, G Sykes, M, Young, C & Krakoff, I* Phase I trial of the podophyllotoxin derivative VM 26 Proc Amer Ass. Cancer Res. 11 79 1970

25 *Venditti J M* Treatment schedule dependency of experimentally active antileukemic (L1210) drugs Cancer Chemother Rep 2 35-59 1971

Acta path microbiol scand Section A 81, 5, 725-733, 1973

THE EFFECT ON RENAL HYPERTENSION OF SUBCUTANEOUS ISOTRANSPLANTATION OF RENAL MEDULLA FROM NORMAL OR HYPERTENSIVE RATS

Including Studies on Spontaneous Variations in Blood Pressure in Normal and Hypertensive Rats

TOVE MANTHORPE

The University Institute for Experimental Medicine, Norre Alle 71,
2100 Copenhagen Ø, Denmark

Following subcutaneous transplantation of normal renal medulla to renal hypertensive rats there was a long lasting depression of the blood pressure There was no further depression after a second transplantation Transplantation of medulla from the ischemic kidney of renal hypertensive donors, produced a similar depression of the blood pressure, whereas medulla from the untouched kidney had little effect Subcutaneous implantation of gelatine sponge sham operation and transplantation of normal renal cortex all produced only short lasting depressions of the blood pressure Blood pressure measured in the afternoon was significantly lower than in the morning, both in normal and in renal hypertensive rats The higher the blood pressure level, the more marked was this absolute difference

In 1958, *Muirhead & Stirman* first focused attention on the renal medulla as a possible site for an antihypertensive factor Since that report, the theory has found support in two experimental situations 1) renomedullary extracts are active in lowering the blood pressure in renoprival and renal hypertension, *Muirhead et al* (1960 and 1966), *Hickler et al* (1965), *Lee et al* (1965) and *Milliez et al* (1965), and 2) isotransplantation of living renal medulla lowers the blood pressure in one kidney Goldblatt (*Muirhead et al* 1970) and in "post salt" (*Tobian & Azar* 1972) hypertensive rats, while transplantation of renal cortex or lyophilized renal medulla is without effect (*Muirhead et al* 1970)

The primary aim of the present study was to see if the findings of *Muirhead* and collaborators could be confirmed Transplantation of renal medulla or cortex was controlled with either s c implantation of gelatine sponge or sham operation It was further investigated if transplantation of renal medulla from either the ischemic or the untouched kidneys of Goldblatt hypertensive rats would have the same effect as transplantation of normal renal medulla As a basis for such a study it was essential to investigate how the blood pressure of the normal and the hypertensive rat varied both within the day and from day to day

Received 18 iv 73 Accepted 18 iv 73

MATERIAL AND METHODS

Animals Female S P F Wistar rats, weighing about 180 g and inbred female S P F Lister rats

weighing about 170 g were fed commercial chow (Rostock pills containing 0 3 *per cent* NaCl) and offered tap water ad libitum *Blood pressure* was measured in the unanaesthetized animal using the tail plethysmographic method (*Williams et al* 1939) Each measurement was obtained by taking the average of between 5 and 10 consecutive readings *Hypertension* was provoked by placing a 0 20 mm narrow silver clip on the left renal artery (*Wilson & Byrom* 1939), the right kidney being left untouched For this procedure, rats were pretreated with 22,500 I U dipenicillin LEO® i m. per 100 g body weight and briefly anaesthetized with ether Hypertensive rats were divided into three groups, those receiving transplantations of medulla, those receiving cortex, and unoperated controls *Selection* into hypertensive groups was performed in such a way that there was a similar degree of hypertension in the three groups *Donor* rats were anaesthetized with pentymal sodium (10 mg i p /100 g), the recipients with ether both being pretreated with penicillin From normal donors renal cortices from 4 kidneys (1 g) and renal medullae from 6 kidneys (0 55 g) were prepared for each transplantation After the capsule had been removed, 2 mm thick sections were cut perpendicular to the long axis of the kidney and the inner and outer sections of the medulla (white and pink portions) were separated from the cortex (brown portion) with fresh surgical blades As quickly as possible the different parts were placed in Tissue Culture Medium (TC 199® Difco) in a sterile container in an icebath When the necessary amount of tissue had been collected the pieces were cut with small scissors into 1 × 1 × 1 mm fragments These fragments were injected subcutaneously into the shaved, iodine alcohol painted ventral abdominal wall using a 14 gauge 2 inch needle introduced to the hub and slowly withdrawn during the injection Two to four tracts were made and any tissue remains in the syringe were injected with 0 5 to 2 5 ml TC 199 In a few cases 3–4 drops of the TC 199, in which the tissue fragments were suspended were transferred to an inoculation stick for bacteriological investigations. None of the inoculations gave any bacterial growth. With transplantation of medulla from either ischemic or the untouched kidneys identical techniques were employed except that the recipients received medullae from either four ischemic or four untouched kidneys the total amount being 0 2 to 0 3 g and 0 3 to 0 4 respectively The time taken from the removal of the first donor kidney to the end of the injection of the fragments was 20 30 minutes When transplants were to be removed animals were placed under light ether anaesthesia a midline incision was made in the ventral abdominal wall the skin flaps undermined and retracted and the tracts of transplanted tissue exposed and completely removed Sub-

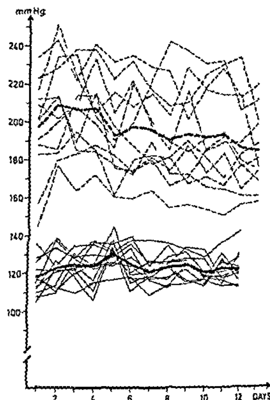


Fig 1 Spontaneous changes in systolic arterial blood pressure (mm Hg) from day to day in normal (———) and hypertensive (– –) rats. The thicker lines define the daily means of the two groups

cutaneous implantation of gelatine sponge (Spongostan Special®) and the sham operations were performed in rats pretreated in the same manner as the experimental groups The 5 cm × 1 cm × 1 mm piece of the sponge was cut into pieces, soaked in TC 199 and placed in s c channels *Statistical evaluation* The differences between the groups were analyzed using the Student s *t distribution*.

RESULTS

Variation in Blood Pressure from Day to Day

Systolic blood pressure for individual rats measured at the same time (± 30 minutes) of the day showed great variation from day to day Fig 1 gives the results of studies on 12 renal hypertensive and 12 normal rats The variation was more pronounced in the hypertensive than in the normal rats, but variations were asynchronous and were large

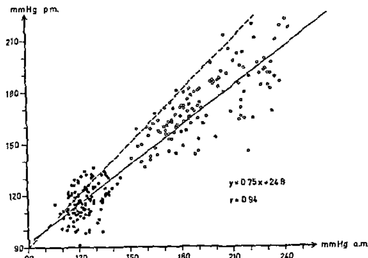


Fig 2 Systolic arterial blood pressure (mm Hg) in normal (●) and hypertensive (O) rats measured in the morning (abscissa) and afternoon (ordinate) Each point is the average of 10 morning and 10 afternoon readings obtained on the same day in the one rat The lines of identity (- -) and regression (——) are shown

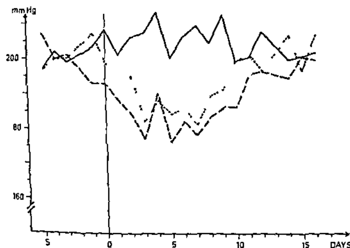


Fig 3 Constancy of the blood pressure in the non operated control group (—— n 6) contrasted with the depression of the blood pressure after the implantation at day O of gelatine sponge (— — — n 6) or after sham operation at day O (n 6)

ly eliminated in the mean values for the groups

Variation in Blood Pressure during the Day

The morning (8 30–9 45 a m) and the afternoon (1 45–3 00 p m) blood pressures were measured during 10 days in 12 normal and in 12 renal hypertensive rats In Fig 2 the morning values are plotted against the afternoon values obtained on the same day The morning was higher than the corresponding afternoon value in almost all cases The absolute difference was more pronounced in the hypertensive than in the normal rats but the relative fall differed only slightly from rat to rat (correlation coefficient $r = 0\ 94$ $P < 0\ 001$ coefficient of regression – 0 75, $P < 0\ 001$) The mean of all the *normal* blood pressure values on all days was $124 \pm 0\ 7$ mm Hg in the morning and $117 \pm 0\ 9$ mm Hg in the afternoon (mean ± S E M) In *hypertensive* animals the values were $194 \pm 2\ 1$ and $172 \pm 1\ 8$ mm Hg respectively Both these differences are highly significant ($P < 0\ 001$) Variance due to the rat itself and to the day of measurement were analyzed by the two-way classification of *Snedecor & Cochran* (1967) for the observed differences in each rat No significant differences emerged in normal rats but in the

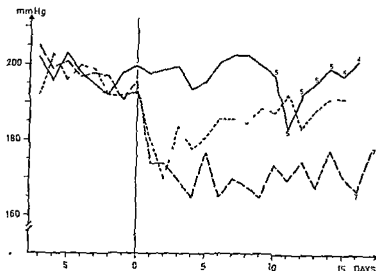


Fig 4 Constancy of the blood pressure in the non operated *control* group (——, n = 6, unless otherwise marked) contrasted with the depression of the blood pressure after the transplantation at day O of normal renal cortex (, *n* = 8) or of normal renal medulla (– – – –, n = 8, unless otherwise marked)

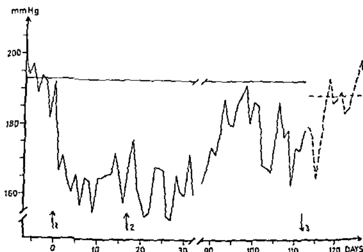


Fig 5 Depression of systolic arterial blood pressure following the first transplantation of normal renal medulla on day O, (marked *by* ↑ *1*), and the lack of further depression following the second transplantation on day 17, (marked by ↑ 2) Both transplants were removed on day 112 (marked by ↓3) One rat died under anaesthesia, thus being shown on the curve by changing from —— (*n* = 5) *to* – – – (n = 4) The horizontal lines indicate the pre transplantation level of the blood ... re in the 5 (——, 193 mm ... and finally 4 rats (– – –, ...3 mm Hg)

hypertensive group the difference *from rat* to rat but not from day to day was significant (F_{rat} = 4 47 i e P < 0 01)

Effect of Subcutaneous Implantation of Gelatine Sponge (Spongostan Special®) and of Sham Operation

There was a slight and significant fall in systolic arterial pressure during the first 9 days both after application of gelatine sponge (from 199 ± 5 0 mm Hg to 183 ± 4 0 mm Hg, mean ± S E M, P < 0 001) and after sham operation (from 201 ± 5 9 mm Hg to 188 ± 3 2 *mm Hg* P < 0 002) as compared with the non-operated controls (202 ± 5 2 mm Hg and 206 ± 4 3 mm Hg) (Fig 3) In the second postoperative period (days 10–16) pressure had returned to the *pre-operative* control level and the mean values for the 3 groups were not different The mean values were, 196 ± 5 2 mm Hg for the gelatine sponge group, 200 ± 5 4 mm Hg for the sham operated and 201 ± 4 5 mm Hg for the non-operated controls None of the rats showed any sign of wound infection

Effect of Subcutaneous Transplantation of Renal Medulla or Cortex from Normal Donors

Fig 4 shows that both medullary and cortical transplantation result in a marked fall in blood pressure, but that after the third day

TABLE 1 *Systolic Arterial Blood Pressure in Renal Hypertensive Rats before and after Transplantations of Normal Renal Medulla and after Removal of the Transplants*

Days	Before the transplantations	After the first transplantation	After the second transplantation		After removal of transplants	
	—7–0	1–17	18–48	49–112	113–128	129 164
mm Hg Mean ± S E M	193 ± 2 6	164 ± 2.3	163 ± 1 8	176 ± 1 4	184 ± 2 1	190 ± 1 0
Number of rats	5	5	5	5	4	3

pressure remained at the low level only in the medulla transplanted group Before the transplantation there were no differences between the three groups (198 ± 1 9 mm Hg, 196 ± 2 3 mm Hg and 198 ± 1 8 mm Hg, means ± S E M). In the first postoperative period days 1–8 the blood pressure (170 ± 2 8 mm Hg) in the medulla transplanted group was significantly lower ($P < 0 005$) than in the cortex transplanted group (181 ± 2 5 mm Hg), which again was significantly lower ($P < 0 001$) than the non-operated controls (199 ± 1 6 mm Hg) In the second postoperative period (days 9–17) the medulla transplanted group still displayed significantly lower ($P < 0 001$) blood pressure (172 ± 2 8 mm Hg) than the cortex transplanted group (189 ± 2 6 mm Hg) and the non-operated controls (196 ± 3 2 mm Hg) the difference between the latter two groups was not significant ($P < 0 2$)

Effect of a Second Transplantation of Normal Renal Medulla

On the 17th day following the first transplantation six of the eight rats which had been previously transplanted with renal medulla, received the same amount of normal renal medulla by a second transplantation One of the rats died on the third day after the second transplantation In Fig 5 the continuous line is the average of the blood pressures of the five rats which survived the second transplantation period It can be seen that, whereas the first transplantation provoked a pronounced fall in blood pressure, there was no further fall following the second transplantation Table 1 shows the mean of the blood pressure values ± S E M and the surviving number of rats during the periods 1) before, 2) after the first transplantation, 3) the first 31 days and 4) the following 64 days after the second transplantation, and finally 5) the first 16 and 6) the following 35 days after removal of the transplants These periods were chosen because the daily average of blood pressures showed no constant variation within these periods It has statistically been shown for all of the individual rats that all variations within these periods were essentially at random 30 days following the second transplantation there was a sudden unexplained rise (during the course of two days) in blood pressure (to 176 mm Hg) which did not however reach the pretransplantation level of 193 mm Hg

Effect of Removal of the Medullary Transplants

On the 112th day after the first transplantation both transplants were removed (Fig 5) One of the 5 rats died under anaesthesia The others showed a rise in blood pressure and all reached the pretransplantation level within the first 6 days after the removal One rat died on the 16th day the others survived and had a blood pressure n[illegible] slightly above the pretransplantation l[illegible] until they were sacrificed on day 52 [illegible] the removal of transplants

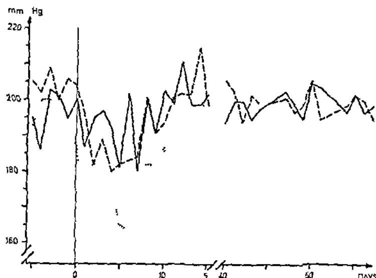


Fig 6 Spontaneous changes in blood pressure in the non trans planted control group (——, n = 3) contrasted with the long lasting depression of the pressure after the transplantation at day O of medulla from the ischemic kidneys of renal hypertensive rats (n = 4) and the short lasting depressive tendency after the transplantation at day O of medulla from the untouched kidneys (– – –, n = 4)

TABLE 2 *Systolic Arterial Blood Pressure in Goldblatt Two Kidney Hypertensive Rats before and after Transplantation of Medulla from either Ischemic or Untouched Kidneys of Hypertensive Donors For Comparison the Blood Pressure of Non Transplanted Control Rats is Given*

Group	Rat no	Medullary transplants from the	Before the transplantation Days —→0 BP mm Hg	Group mean BP mm Hg	After the transplantation Days 3 21 Change in BP mm Hg	Group mean BP mm Hg	Days 26 57 Change in BP mm Hg	Group mean BP mm Hg
	96	Ischemic kidneys	197 ± 4 0	193 ± 3 1	−12 ± 5 9	180 ± 2 5	33 ± 4 5	167 ± 1 1
	104		190 ± 2 8		25 ± 4 7		28 ± 3 0	
	106		197 ± 10 1		16 ± 11 3		29 ± 10 3	
	108		188 ± 6 4		0 ± 8 1		12 ± 6 8	
B	94	Untouched kidneys	196 ± 5 7	204 ± 3 6	8 ± 7 1	195 ± 2 7	3 ± 6 4	200 ± 1
	95		208 ± 11 4		3 ± 12 7		1 ± 11 9	
	99		206 ± 5 3		2 ± 6 5		−1 ± 5 7	
	100		208 ± 4 8		31 ± 6 9		18 ± 5 2	
C	91	Non transplanted controls	199 ± 3 3	197 ± 2 6	5 ± 4 1	199 ± 2 7	5 ± 3 5	199 ± 1
	101		189 ± 2 7		−10 ± 4 0		1 ± 3 7	
	123		202 ± 5 9		12 ± 7 0		13 ± 6 3	

BP blood pressure expressed as mean ± S E M

Effect of Transplantation with Renal Medulla either from the Ischemic or from the Untouched Kidney to a Limited Number of Recipients

The results are shown in Fig 6 and Table 2 During the first 3–4 weeks after the trans plantation, the group mean blood pressure showed greater variations in all 3 groups than in any other period (Only the values from the first 15 days are shown in Fig 6) In spite of this variation it is evident that the fall in blood pressure in group A (ischemic) was more long lasting than that in group B (untouched) From the end of the 4th week the blood pressures in group B (untouched) and group C (control) were more stable and at the same level (∼ 200 mm Hg) as before the transplantation whereas in group A

(ischemic) pressure was more stable and at a significantly ($p < 0.001$) lower level (167 mm Hg) Only the values from the last 18 days of experiment are shown in Fig 6

DISCUSSION

1 Variation of Blood Pressure in Untreated Normal and Renal Hypertensive Rats

Marked variations in day to day blood pressure have been found by other investigators (*Bing* 1962, *Reichle* 1971 and *Frohlich et al* 1972), but it does not seem to have been noticed that there is a significant fall in blood pressure during the day in both normal and hypertensive rats On the contrary, *Reichle* (1971) found the difference between morning and afternoon values insignificant In the hypertensive group it was found that the more pronounced the hyper tension, the greater was the absolute fall Such a fall must be taken into account in experiments of the present type where blood pressure is followed from day to day, and in experiments where blood pressure is measured within the one day

2 Effect of Sham Operation, of Implantation of Gelatine Sponge and of Transplantation of Renal Cortex

The fall in blood pressure during the first 9 days after these operations is probably a non specific effect following the injury of the tissues This depression could be the result of a prostaglandin release, as it has been shown that mechanical stimulation of various tissues provokes such a release (*Palmer et al* 1970 and *Gryglewski* & *Vane* 1972) The fall seen after transplantation of renal cortex (Fig 4) to two-kidney renal hypertensive rats (one renal artery clipped, the opposite kidney untouched) was of the same duration and degree, as the fall seen after implantation of gelatine sponge or sham operation (Fig 3) and can therefore also be considered non specific. This depressor effect contrasts with the result of experiments of *Muirhead et al* (1970), who found a slight but significant rise after cortical transplantation to one kidney-renal hypertensive rats (one renal artery clipped, the opposite kidney removed)

3 Effect of Transplantation of Normal Renal Medulla

The depression in blood pressure seen after transplantation of normal medulla (Fig 4) was both greater and more prolonged than those seen after the three previous mentioned operations Although the initial hypertensive blood pressure in this experiment was considerably higher than that in the experiments of *Muirhead et al* (1970) ($\simeq$ 200 mm Hg versus $\simeq$ 170 mm Hg) the absolute fall in blood pressure was the same ($\sim$ 28 mm Hg) This, together with the fact that in this experiment there was no relation between the blood pressure level before and the size of the fall after the transplantation in the individual rats, indicates that this fall—in contrast to that seen within single days—is independent of the degree of hypertension Such independence has also been found by *Muirhead* (personal communication)

The lack of further depressor effect of a *second transplantation* indicates that the maximal possible effect of transplantation has been achieved by giving 0 55 g medullary tissue This amount was slightly more than used by *Muirhead et al* (1970), who achieved the same absolute fall in blood pressure by giving 0 4 g During the last month before the removal of the transplants the mean blood pressure curve (Fig 5) showed much greater variation from day to day than was the case previously This was mainly due to synchronous variations in the individual rats The tendency to rise in blood pressure in the few experiments with removal of the trans plants is in agreement with the results of *Muirhead et al* (1970)

4 Effect of Transplantation of Renal Medulla from Hypertensive Rats

It is generally assumed that the presence of the untouched kidney is the reason for both the more benign hypertension in the two

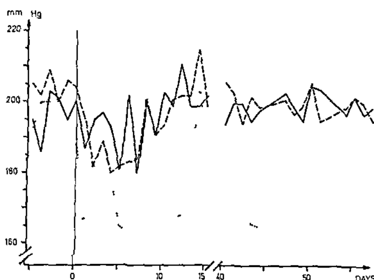


Fig 6 Spontaneous changes in blood pressure in the non transplanted control group (——, n = 3) contrasted with the long lasting depression of the pressure after the transplantation at day O of medulla from the ischemic kidneys of renal hypertensive rats (, n = 4), and the short lasting depressive tendency after the *transplantation at day O of* medulla from the untouched kidneys (– – –, n = 4)

TABLE 2 *Systolic Arterial Blood Pressure in Goldblatt Two Kidney Hypertensive Rats before and after Transplantation of Medulla from either Ischemic or Untouched Kidneys of Hypertensive Donors For Comparison the Blood Pressure of Non-Transplanted Control Rats is Given*

Group	Rat no	Medullary transplants *from the*	Before the transplantation Days —5–0 BP mm Hg	Before the transplantation Days —5–0 Group mean BP mm Hg	After the transplantation Days 3 21 Change in BP mm Hg	After the transplantation Days 3 21 Group mean BP mm Hg	After the transplantation Days 26–57 Change in BP mm Hg	After the transplantation Days 26–57 Group mean BP mm Hg
	96		197 ± 4 0		–12 ± 5 9		33 ± 4 5	
	104	Ischemic	190 ± 2 8		25 ± 4 7		28 ± 3 0	
	106	kidneys	197 ± 10 1	193 ± 3 1	16 ± 11 3	180 ± 2 5	–29 ± 10 3	167 ± 1 1
	108		188 ± 6 4		0 ± 8 1		–12 ± 6 8	
	94		196 ± 5 7		–8 ± 7 1		3 ± 6 4	
	95	Untouched	208 ± 11 4		–3 ± 12 7		–1 ± 11 9	
B	99	kidneys	206 ± 5 3	204 ± 3 6	2 ± 6 5	195 ± 2 7	–1 ± 5 7	200 ± 1 4
	100		208 ± 4 8		31 ± 6 9		18 ± 5 2	
	91	Non	199 ± 3 3		5 ± 4 1		5 ± 3 5	
C	101	transplanted	189 ± 2 7	197 ± 2 6	10 ± 4 0	199 ± 2 7	1 ± 3 7	199 ± 1 7
	123	controls	202 ± 5 9		12 ± 7 0		13 ± 6 3	

BP blood pressure expressed as mean ± S E M

Effect of Transplantation with Renal Medulla either from the Ischemic or from the Untouched Kidney to a Limited Number of Recipients

The results are shown in Fig 6 and Table 2 During the first 3–4 weeks after the transplantation, the group mean blood pressure showed greater variations in all 3 groups than in any other period (Only the values from the first 15 days are shown in Fig 6) In spite of this variation it is evident that the fall in blood pressure in group A (ischemic) was more long-lasting than that in group B (untouched) From the end of the 4th week the blood pressures in group B (*untouched*) and group C (*control*) were more stable and at the same level (~ 200 mm Hg) as before the transplantation, whereas in group A

15 *Muirhead, E E* & *Sturman, J A* Dietary protein and hypertension of dog Protection by uretero-caval anastomosis with a study of kidneys so treated Am J Path. *34* 561–562, 1958
16 *Palmer, M A*, *Piper, P J* & *Vane, J R* The release of rabbit aorta contracting substance (RCS) from chopped lung and its antagonism by anti inflammatory drugs Br J Pharmacol *40* 581P 582P, 1970
17 *Reichle, F A* Noninvasive blood pressure measurement in the rat by ultrasonic auscultation J Surg Res *11* 539–544, 1971
18 *Sen, S*, *Smeby, R R* & *Bumpus, F M* Antihypertensive effect of an isolated phospholipid Am J Physiol *214* 337–341, 1968
19 *Snedecor, G W* & *Cochran, W G* Statistical Methods 6th ed The Iowa State University Press Ames, Iowa 1967, pp 299–301
20 *Strong, C G*, *Boucher, R*, *Nowaczynski, W* & *Genest, J* Renal Vasodepressor Lipid Mayo Clin Proc *41* 433–452, 1966
21 *Tobian, L* & *Azar, S* Antihypertensive action of the renal papilla In Genest J & Koiw, E (Ed) Hypertension '72, 1 ed Springer Verlag, Berlin, Heidelberg and New York 1972 p 393–399
22 *Williams, J R*, *Harrison, T R* & *Grollman, A* A simple method for determining the systolic blood pressure of the unanesthetized rat J Clin Invest *18* 373 376, 1939
23 *Wilson, C* & *Byrom, F B* Renal changes in malignant hypertension Lancet *1* 136–139, 1939

Acta path microbiol scand Section A 81, 5, 734–736, 1973

BRIEF REPORT

THE ULTRASTRUCTURE OF CONTACT ZONES BETWEEN PLASMA CELLS AND DENDRITIC MACROPHAGES FROM PATIENTS WITH MULTIPLE MYELOMA

Jens Blom

A small number of plasma cells of sternal bone marrow aspirations from five patients with IgG multiple myeloma were found to be in direct contact with cytoplasmic processes of dendritic macrophages In these contact zones a characteristic thickening of the inner leaflet of the cytoplasmic membrane of the plasma cell was observed (Figs 1 & 2)

The preparative procedures used were as follows The sternal bone marrow aspirations were immediately injected into three per cent glutaraldehyde buffered to pH 7 2 with 0 1 M sodium cacodylate (11) and allowed to stand for 1 hour at room temperature (20–23° C) The clumps of clotted cells were then cut into 1 mm^3 cubes and fixation was continued in fresh fixative of the same osmolality for another 4 hours After transfer to 0 2 M sucrose in 0 1 M cacodylate buffer pH 7 2 and storage overnight at 4° C, the specimens were postfixed for 1 hour at room temperature in 1 per barbiturate buffered OsO_4 pH 7 2 with 4 5 per cent sucrose added (1) The specimens were then treated en bloc for 1 hour with 2 per cent uranyl acetate in the same buffer without sucrose, dehydrated in alcohol and propylene oxide (6) and finally embedded in Vestopal W (10) Sections for electron microscopy were post stained with magnesium uranyl acetate (3) and lead citrate (9)

The macrophages showed no thickening of the plasma membranes in the contact zones These were characterized by gaps of about 20 nm between apposing cell membranes and delicate electron opaque bridges were noted across the gaps (Fig 1) The additional membrane layer of the plasma cell corresponding to the contact zones varied in thickness from 18 25 nm (Fig 3) It was characteristic that the cytoplasm adjacent to these zones was sparse in organelles except for cytoplasmic fibrils (Figs 1 & 3) The changes described were only observed in plasma cells, but otherwise these plasma cells did not differ in morphology from the other plasma cells of the bone marrow The macrophages normally had an irregular nucleus and the cytoplasm ramified into long, complex processes which intertwined between the other cells of the bone marrow (Fig 2) In the cytoplasm ferritin particles were often seen (Fig 1) whereas very few phagolysosomes were observed The length of the contact zones with the plasma cells were found to vary from 200 nm to 1–2 μm in the sections studied, and often a plasma cell had several contact sites with one macrophage

These contact zones, comprising thickening of the plasma membrane of the plasma cell, were observed in specimens taken prior to and after treatment of the patients with melphalan or cyclophosphamide. At present however, the function of these zones is unknown Recent publications (2, 5, 7, 8, 12, 13) have demonstrated that a collaboration between

Received 24 vii 73 Accepted 24 vii 73

Department of Biophysics Statens Seruminstitut Copenhagen, Denmark

Requests for reprints should be addressed to Dr J Blom, Department of Biophysics, Statens Seruminstitut, Amager Boulevard 80 DK 2300 Copenhagen S, Denmark

Fig 1 The contact zone between a plasma cell (PC) and a dendritic macrophage (DM) (Fe) denotes ferritin particles in the macrophage cytoplasm 90 000 ×

Fig 2 Part of a plasma cell (PC) with dilated rough endoplasmic reticulum (RER) and a dendritic macrophage (DM) The arrow points to a characteristic contact zone. 15,000 ×

Fig 3 Detail from Fig 2 A part of the macrophage nucleus (MN) is seen The additional layer [illegible]

90 000 ×



MN
DM
PC
DM
PC
RER
2

thymus dependent (T) and bone marrow derived (B) lymphocytes seems to be necessary for elicitation of an antibody response to many antigens, and that this interaction also requires the participation of dendritic macrophages In an ultrastructural study of antigen binding sites on rosette forming cells (4), it was shown that an electron dense material was present along the inner aspect of the plasma membrane of some lymphocytes corresponding to the antigen binding immunoglobulin receptors on the surface of these lymphocytes. With these results in mind, it is tempting to speculate whether the contact sites described in this paper could be areas where antigen antibody complexes are bound to the surface of dendritic macrophages Contact of these complexes with B lymphocytes might stimulate these cells to proliferate and thus give rise to antibody producing plasma cells On the other hand, contact between dendritic macrophages and lymphocytes of the bone marrow has not been observed in the present material

However, multiple myeloma is best defined as a malignant proliferation of plasma cells (14), and thus the observed contact zones between plasma cells and dendritic macrophages could constitute a feature of this abnormal plasma cell proliferation Further work is in progress to characterize the contact zones by immunological methods

References 1 *Caulfield, J B* J biophys. biochem Cytol *3* 827–830, 1957 —2 *Feldmann, M* J exp Med *135* 1049–1058, 1972 —3 *Frasca, J M* & *Parks, V R* J Cell Biol *25* 157–161, 1965 —4 *Gudat, F G* & *Villiger, W* J exp Med *137* 483–493, 1973 —5 *Lachmann, P J* Proc roy Soc B, *176* 425–426, 1971 —6 *Luft, J H* J biophys biochem Cytol *9* 409–414, 1961 —7 *Mitchison, N A* Immunology *16* 1–14, 1969 —8 *Nossal, G J V*, *Abbot, A*, *Mitchell, J* & *Lummus, Z* J exp Med *127* 277–290, 1968 —9 *Reynolds, E S* J Cell Biol *17* 208–212 1963 —10 *Ryter, A* & *Kellenberger, E* J Ultrastruct Res *2* 200–214, 1958 —11 *Sabattini, D D*, *Bensch, K* & *Barnett, R J* J Cell Biol *17* 19–58, 1963 —12 *Taussig, M J* & *Lachmann, P J* Immunology, *22* 185–197, 1972 —13 *Taylor, R B* & *Iverson, G M* Proc. roy Soc. B, *176* 393–418, 1971 —14 *Waldenstrom, J* Diagnosis and treatment of multiple myeloma Grune & Stratton, New York 1970

Acta path. microbiol scand Section A 81, 737–750 1973

ENZYME HISTOCHEMISTRY OF THE LIVER IN EXTRAHEPATIC BILIARY OBSTRUCTION

A Comparison between Man, Dog and Rat

I Hagerstrand

The Institute of Pathology, the Histochemical Department, Malmo General Hospital
University of Lund, Malmo, Sweden

Morphological and enzyme histochemical changes during extrahepatic biliary obstruction in the liver tissue of man, dog and rat were compared The differences in morphological changes were mainly quantitative. Ductular proliferation was less marked and occurred later in livers of man and dog than in rat liver and there were more cellular changes centrolobularly in the livers of the former two species The qualitative differences consisted of lack of cholestasis in the rat liver and of focal necroses in the dog liver Staining for succinic dehydrogenase and glucose 6 phosphatase showed decreased activity while lactic dehydrogenase and mono-amine oxidase remained unchanged The lysosomal enzyme activities (acid phosphatase and beta glucuronidase) were increased in hepatocytes and Kupffer cells but the increase was most evident in connection with biliary pigment in livers of dog and man The membrane-associated hydrolases (adenosine triphosphatase alkaline phosphatase leucine aminopeptidase gamma glutamyl transpeptidase) varied in the three species with alternating increase and decrease at the sinusoids and canaliculi The findings are discussed with reference to ultrastructural and biochemical changes during extrahepatic biliary obstruction The serum activities of alkaline phosphatase and gamma glutamyl transpeptidase during biliary obstruction in the three species are reported

The enzyme histochemical changes of the liver during extrahepatic biliary obstruction have been the subject of earlier reports (1, 2, 18) The works concerned livers from the rat, the dog and man In the present paper the changes in the three species will be compared followed by a discussion of the histochemistry concerning the biochemical and ultrastructural hepatic changes during biliary obstruction Remarks on serum activities of alkaline phosphatase and gamma glutamyl transpeptidase during extrahepatic biliary obstruction will be included

Received 4 iv 73 Accepted 5 v 73

MATERIAL AND METHODS

The common bile duct was ligated in 9 dogs and 81 albino rats Liver tissue and blood samples were studied before during and after the release of the occlusion The occlusion was maintained for 4 weeks in the dogs and from 4 hours to 7 weeks in the rats Twenty-eight surgical liver biopsy specimens from patients in whom the common bile duct had been obstructed for varying periods (7 days to 4 years) were examined The specimens were stained for activities of the following enzymes succinic dehydrogenase mono-amine oxidase glucose-6 phosphatase lactic dehydrogenase non specific esterase acid phosphatase beta glucuronidase alkaline phosphatase adenosine triphosphatase leucine aminopeptidase gamma glutamyl transpeptidase

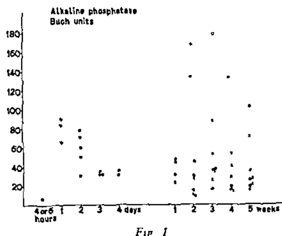


Fig 1

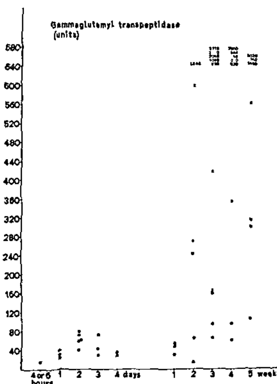


Fig 2

Fig 1 and 2 Serum activities of alkaline phosphatase and gamma glutamyl transpeptidase during the course of biliary obstruction Human cases are marked by crosses in fig 2 partly by numerals

Dogs are marked by rings

Rats are marked by dots

Normal values for alkaline phosphatase and gamma glutamyl transpeptidase in man 2 8 and 1·25 units, in dog 5 7 ± 2 4 and 14 8 ± 3 8 units, in rat 15 ± 14 and 12 ± 15 units

> values higher than

The material and methods used have been described in detail previously (1, 2, 18) The material included also 1 dog and 8 rats not reported earlier, in these animals, the common bile duct had been ligated for 6 months and for 4 hours to 10 days, respectively

RESULTS

Morphological Comparison

The changes in the human livers resembled those in the dog livers more closely than those in the rat livers The rat livers did not show bile pigment or casts and except for focal necroses (Charcot-Gombault, 18) in the early phase of biliary obstruction (1st to 5th day), the changes were confined to the portal tracts with pronounced proliferation of bile ducts and ductules In man and dog, the changes were less pronounced in the portal tract while centrolobular changes with bile deposits fatty changes and feathery degeneration of hepatocytes were striking In human cases of protracted icterus (1 month's duration), groups of damaged and necrotic, bile stained hepatocytes were sometimes seen The dog livers disclosed no changes resembling the Charcot-Gombault necroses of the rat livers or the necroses of the human livers (Fig 3 a d)

Comparison of Enzyme Histochemistry and Serum Enzyme Activities

The activity of the mitochondrial enzyme *succinic dehydrogenase* appeared to be decreased throughout biliary obstruction in all

---

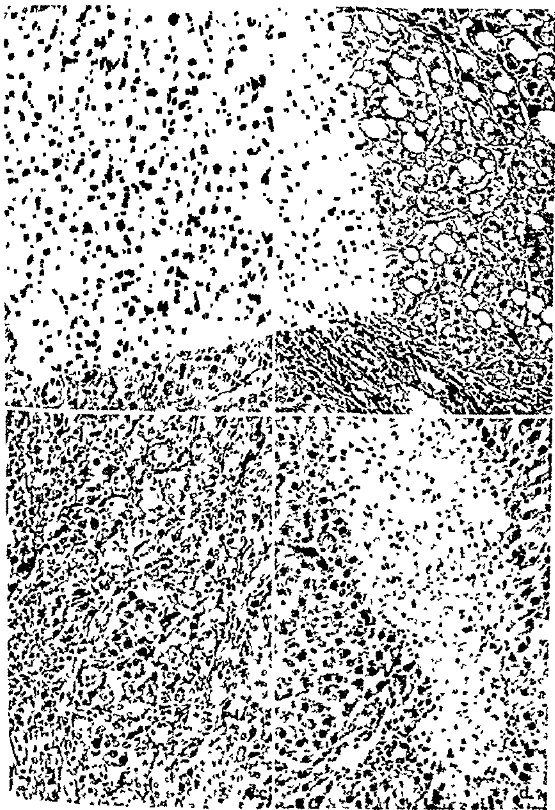
Fig 3 a Γ 1501/68 Human liver Four weeks of jaundice Part of portal zone at the bottom with two small bile ducts to the left Bile casts (marked by arrows) and swollen hepatocytes

b Dog liver after 4 weeks obstruction of the common bile duct Fatty change At least one, probably new formed bile duct in the lower right corner (marked by arrow)

c Rat liver 4 weeks' obstruction Pronounced ductular proliferation with disintegrated liver cell plates.

d Rat liver 1 day s obstruction Charcot Gombault necrosis

htx-e × 178




a
b
c
d

three species In human livers, cells adjacent to bile material sometimes showed increased activity with densely packed, finely granular deposits

Staining for *mono amine oxidase and lactic dehydrogenase* gave similar results in all three species and no obvious changes occurred during common bile duct obstruction Stainings for *glucose 6 phosphatase* normally produced a stronger reaction in the periphery than in the centre of the hepatic lobules in all three species Larger central areas with weak reaction were observed in rat livers after the 3rd day of obstruction and sometimes in human livers Only occasionally, dog livers with this reaction were studied

Non specific esterase showed a similar pattern and no obvious changes in the three species, except for intense staining of hepatocytes around bile deposits in livers of dogs and man

The above described five enzyme reactions resulted in diffuse staining of the cytoplasm of hepatocytes

Acid phosphatase showed a similar pattern in normal liver specimens in the three species Lysosomes appeared as pericanalicular granules in hepatocytes and as coalescent, rather homogeneous lumps in Kupffer cells Large acid phosphatase positive bodies of homogeneous appearance were seen in cholestatic centrolobular areas in livers of dogs and man In the dogs, these bodies appeared whenever bile deposits became more numerous and disappeared earlier than bile deposits The bodies in the dog were round and situated just outside the cholestatic regions while those in human livers were larger, more irregular in outline and more closely related to bile material (Fig 4a, c) In human specimens, branched bile casts depicting canaliculi were sometimes seen within these bodies The origin of these bodies could not be determined with certainty They might represent hepatocytes and/or Kupffer cells or damaged hepatocytes digested by Kupffer cells In some human cases they were related to diastase resistant PAS positive material in the cytoplasm Outside cholestatic regions, the number of pericanalicular granules had decreased in some specimens, increased in others Numerous positive Kupffer cells were sometimes noted in livers of dogs and man After the 4th day of obstruction, the acid phosphatase reaction in rat liver showed increased staining in hepatocytes as well as Kupffer cells (Fig 4d)

Beta glucuronidase showed largely the same pattern in the three species, and the location coincided with that of acid phosphatase However in the normal dog liver, mainly Kupffer cells were positive In the rat, positive Kupffer cells were more numerous during biliary obstruction while the staining reaction in hepatocytes sometimes was decreased Like acid phosphatase beta-glucuronidase in human livers was increased around bile pigment however the product was granular (Fig 4b) A similar increase in the granular reaction product around bile pigment was seen in a dog with biliary cirrhosis (obstruction of the common bile duct for 6 months no globules were seen in acid phosphatase staining of this liver) The specimens from the other dogs were not stained for beta-glucuronidase activity

The site of normal *alkaline phosphatase* activity differed with the species In the dog,

---

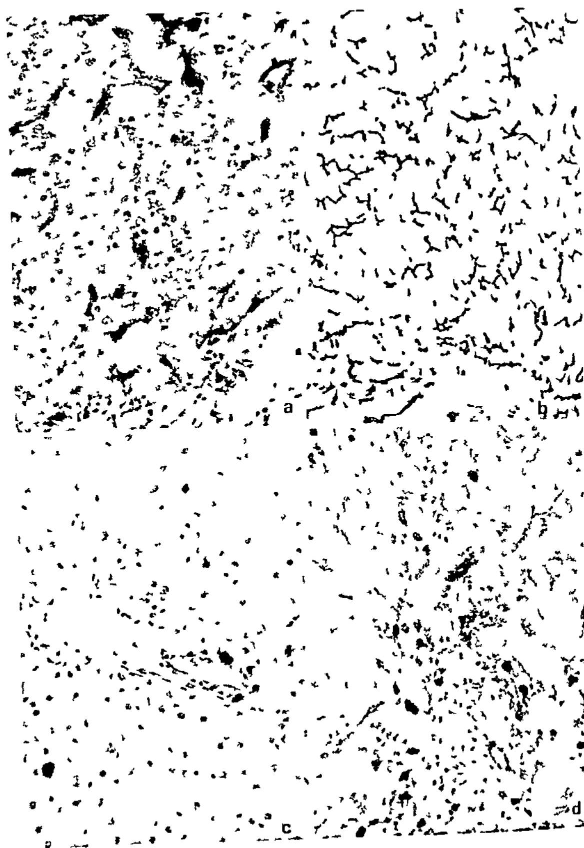
Fig 4 a T 16703/69 Human liver 3 weeks obstruction Centrolobular area in the upper half with bile casts (marked by arrows) and lumps of acid phosphatase reaction products Weak pericanalicular reaction and some positive Kupffer cells in the lower half No counterstaining

b T 5512/69 Human liver 2 weeks obstruction. Dark bile casts surrounded by pale granular reaction product representing beta glucuronidase activity, faint pericanalicular *positivity outside the cholestatic region* No counterstaining

c Dog liver Acid phosphatase positive globules around an area of bile casts (casts marked by arrows) The pale granular strands are mainly lipofuscin faintly positive in the enzyme reaction

d Rat liver 5 days obstruction Strong reaction for acid phosphatase in Kupffer cells and hepatocytes. Note the empty canalicular spaces Faint nuclear counter staining

× 178


a
b
c
d

periportal canaliculi and biliary duct epithelium were stained In the rat, the activity was confined to the portal connective tissue around vessels and biliary ducts, though a faint reaction was sometimes discernible along sinusoids and periportal canaculi In the human liver, the walls of central veins and surrounding sinusoids, periportal arteries and veins and sinusoids next to the limiting plate contained reaction product In the early phase of biliary obstruction, an increased amount of reaction product appeared in cana liculi in livers of dogs and rats and later also in sinusoids In the human livers, the sinusoids showed an early and always more pronounced increase than the canaliculi The latter disclosed positivity only in one fourth of the cases (Fig 5 a-d)

The increase in the *serum alkaline phosphatase* activity was highest in the dog It appeared earliest in the rat where it reached peak values after 1 day of biliary obstruction (see Fig 1)

In normal animals, the staining for *adenosine triphosphatase* activity was similar in all three species and the changes in extra-hepatic biliary obstruction were identical, including loss of canalicular stainability and increased staining of sinusoids, mainly at centrolobular sites (Fig 6 a d)

The site of normal *leucine aminopeptidase* activity varied In the rat, the canaliculi throughout the lobules were seen as slender lines, the portal connective tissue was positive, the picture resembling that of alkaline phosphatase The normal human liver had positive canaliculi—thicker and more knotty than in adenosine triphosphatase staining, but also positive sinusoidal walls, the latter were stronger at peripheral than at centrolobular sites The human bile duct epithelium was positive In the normal dog liver, the Kupffer cells were strongly positive while the bile duct epithelium and canaliculi were negative During biliary obstruction, the human livers showed wide canalicular lumina containing bile casts with strong or negative peptidase reaction of the canalicular walls The sinusoidal activity was sometimes decreased The rat liver showed a decrease in number of positive canaliculi at centrolobular sites, and widened, positive canaliculi at perilobular sites, often with sheets of polyhedric canaliculi, no reaction appeared in the sinusoids Some positive canaliculi as well as positive Kupffer cells were seen in the liver from the dog in which the common bile duct had been ligated for 6 months The reaction was not performed in the other experimental dogs (Fig 7 a-d)

Gamma-glutamyl transpeptidase activity was confined to periportal canaliculi in normal livers of man and dog, while the rat liver did not show any activity or only faint staining of bile duct epithelium. Rats with biliary obstruction showed activity in proliferating ducts and ductules Sometimes the human liver showed activity in canaliculi throughout the lobules as well as in ducts and ductules After prolonged biliary obstruction, the activity would be decreased in the dog

The *serum activity of gamma-glutamyl transpeptidase* in extrahepatic biliary obstruction was highest in man, intermediate in the dog, while the increase was only moderate in the rat (Fig 2)

Fig 5 a T 4800/68 Human liver 4 weeks obstruction due to carcinoma pancreatis Increased sinusoidal activity of alkaline phosphatase Part of portal zone at the bottom Nuclei counterstained

b Dog liver 4 weeks' obstruction Increased canalicular activity No counterstaining

c Control rat liver Positive dots in portal connective tissue and in sinusoids (leucocytes ?) Nuclei counterstained

d Rat liver 5 weeks obstruction Increased numbers of positive dots around proliferating ducts and faint canalicular positivity Nuclei counterstained

Alkaline phosphatase × 178

DISCUSSION

Morphological and Enzyme Histochemical Differences

The differences between the histological hepatic changes in the three species were

essentially quantitative Centrolobular cell damages were most advanced in livers of dogs and man. However, ultrastructural examinations have shown similar changes of hepatocytic mitochondria and smooth reticulum in cholestatic livers of man and rat (8, 23, 25) Ductal proliferation was seen in livers from dog and man, but occurred later and was less advanced than in rat liver This difference remains to be explained Some substances are known to stimulate ductal growth like alpha naphtylisothiocyanate and litocholic acid (22, 27), but the factor acting during common bile duct obstruction is unknown In dogs and rats the ductal growth is limited to liver tissue with occlused extrahepatic ducts as seen in experiments on lobar bile duct obstruction (19, 30) —The qualitative light microscopical differences between the three species were the lack of focal necroses in dog liver (21) and of bile deposits in rat liver The *Charcot-Gombault* necroses of the rat liver are thought to be due to retention of bile in combination with mechanical stasis (16) The necroses seen in the human cases occurred rarely and only in cases of longstanding bile obstruction, it is uncertain whether they have the same aetiology In our material, the human necroses were strongly positive if stained for acid phosphatase, while all the enzyme reactions were totally negative in the rat necroses. This may however, be due to different stages of development Acid phosphatase positivity has been found by *Chou* & *Gibson* (9) in necroses of the rat liver —The lack of bile pigments and bile casts in the rat liver remains to be explained and the same applies to the formation of these in the other species Mechanical bar is not necessary for an occurrence of bile casts We have found casts in non-obstructed liver lobes from dogs subjected to lobar bile duct obstruction (19) and in livers from patients with haemolytic jaundice The composition of the bile most probably accounts for the ability to form bile deposits *Schaffner* & *Popper* (25) have suggested that a disturbance of the micelle formation by the bile salts migth be the cause of bile casts Together with co-workers they recently published results obtained in studies of bile acids in man and rat during biliary obstruction (13, 14) In man, trihydroxylated cholic acid is the main normal bile acid and it is raised by 6-16 times the normal values in icteric patients The dihydroxylated chenodeoxycholic acid was found to be raised in 2 patients with protracted extrahepatic biliary obstruction In the rat, examined up to ten days after common bile duct ligation and excision the trihydroxylated beta muricholic acid increased up to 5 times the normal values, while dihydroxylated bile acids showed no increase Cholic acid was also the main normal bile acid in the rat The authors found a correlation between the concentration of dihydroxylated bile acids and feathery degeneration in human cholestatic livers However, it is still unclear whether the change in bile acid production during biliary obstruction may be the cause of precipitation of bile pigment and bile casts The serum bilirubin increases to equivalent values in the three species (1, 2, 7), but in rats at least, also the production of different bilirubin conjugates is altered during biliary obstruction (32)

It remains unclear whether the absence of gallbladder in the rat is of significance for the different morphological reaction patterns during common bile duct obstruction

Many enzyme reactions showed the same pattern (succinic dehydrogenase, monoamine

---

Fig 6 a T 182/68 Normal human liver demonstrating activity in sinusoids and canaliculi as well as in vessels and bile ducts of a portal zone at the bottom

b T 16703/69 Human liver 3 weeks obstruction Rather distinct, positive sinusoids between fatty degenerated hepatocytes Hardly visible canaliculi (marked by arrows)

c Control rat liver

d Rat liver after 2 weeks obstruction Enzyme reaction product in connection with proliferating bile ducts at the bottom and to the right At the left upper corner hepatocytes with decreased canalicular activity

Adenosine triphosphatase × 178


a
b
c
d

essentially quantitative Centrolobular cell damages were most advanced in livers of dogs and man However, ultrastructural examinations have shown similar changes of hepatocytic mitochondria and smooth reticulum in cholestatic livers of man and rat (8, 23, 25) Ductal proliferation was seen in livers from dog and man, but occurred later and was less advanced than in rat liver This difference remains to be explained Some substances are known to stimulate ductal growth like alpha naphtylisothiocyanate and litocholic acid (22, 27), but the factor acting during common bile duct obstruction is unknown In dogs and rats the ductal growth is limited to liver tissue with occlused extrahepatic ducts as seen in experiments on lobar bile duct obstruction (19, 30) —The qualitative light microscopical differences between the three species were the lack of focal necroses in dog liver (21) and of bile deposits in rat liver The Charcot Gombault necroses of the rat liver are thought to be due to retention of bile in combination with mechanical stasis (16) The necroses seen in the human cases occurred rarely and only in cases of longstanding bile obstruction, it is uncertain whether they have the same aetiology In our material, the human necroses were strongly positive if stained for acid phosphatase while all the enzyme reactions were totally negative in the rat necroses This may however be due to different stages of development Acid phosphatase positivity has been found by *Chou & Gibson* (9) in necroses of the rat liver —The lack of bile pigments and bile casts in the rat liver remains to be explained and the same applies to the formation of these in the other species Mechanical bar is not necessary for an occurrence of bile casts We have found casts in non-obstructed liver lobes from dogs subjected to lobar bile duct obstruction (19) and in livers from patients with haemolytic jaundice The composition of the bile most probably accounts for the ability to form bile deposits *Schaffner & Popper* (25) have suggested that a disturbance of the micelle formation by the bile salts migth be the cause of bile casts Together with co-workers they recently published results obtained in studies of bile acids in man and rat during biliary obstruction (13, 14) In man, trihydroxylated cholic acid is the main normal bile acid and it is raised by 6 16 times the normal values in icteric patients The dihydroxylated chenodeoxycholic acid was found to be raised in 2 patients with protracted extrahepatic biliary obstruction In the rat, examined up to ten days after common bile duct ligation and excision the trihydroxylated beta muricholic acid increased up to 5 times the normal values while dihydroxylated bile acids showed no increase Cholic acid was also the main normal bile acid in the rat The authors found a correlation between the concentration of dihydroxylated bile acids and feathery degeneration in human cholestatic livers However it is still unclear whether the change in bile acid production during biliary obstruction may be the cause of precipitation of bile pigment and bile casts The serum bilirubin increases to equivalent values in the three species (1, 2 7), but in rats at least, also the production of different bilirubin conjugates is altered during biliary obstruction (32)

It remains unclear whether the absence of gallbladder in the rat is of significance for the different morphological reaction patterns during common bile duct obstruction

Many enzyme reactions showed the same pattern (succinic dehydrogenase, monoamine

---

Fig 6 a T 182/68 Normal human liver demonstrating activity in sinusoids and canaliculi as well as in vessels and bile ducts of a portal zone at the bottom

b T 16703/69 Human liver 3 weeks obstruction. Rather distinct positive sinusoids between fatty degenerated hepatocytes Hardly visible canaliculi (marked by arrows)

c Control rat liver

d Rat liver after 2 weeks obstruction Enzyme reaction product in connection with proliferating bile ducts at the bottom and to the right At the left upper corner hepatocytes with decreased canalicular activity

Adenosine triphosphatase × 178

oxidase, lactic dehydrogenase glucose-6 phosphatase, adenosine triphosphatase), *others* differed more or less in the three species whether during normal conditions or during biliary obstruction The most striking diversity was found in the case of alkaline *phosphatase activity which in the human* liver was localized to the sinusoids and seldom disclosed canalicular activity during biliary obstruction Strong canalicular activity was present in the dog liver, being weak in the *rat liver during biliary obstruction However*, the increased alkaline phosphatase activity in the dog series was partly due to barbiturate anaesthesia (3) Such factors have apparently no influence on the rat and man —Leucine aminopeptidase differed in location and intensity in the three species under normal *conditions (Fig 7)* A decreased canalicular activity occurred at centrolobular sites in rats, while human livers showed increase or decrease around bile casts and sometimes decreased activity in the sinusoids —Gamma-glutamyl *transpeptidase is an enzyme of the* biliary passages in all three species, localized to periportal canaliculi in livers of dogs and man, but localized to ducts and ductules in livers of the rat Only human livers disclosed increased canalicular activity during biliary obstruction

Biliary Obstruction and Oxido Reductases and Glycogen

Sections stained for oxido reductase activities were largely of normal appearance, but suggested a decrease in *succinic dehydrogenase* Likewise, glucose 6 phosphatase activity seemed to be diminished In some human *livers, scattered hepatocytes in areas with* biliary pigment were strongly positive for succinic dehydrogenase activity This activity might correspond to the ultrastructural finding of hepatocytes with closely packed mito*chondria, perhaps due to a decrease of rough* endoplasmic reticulum and glycogen (8) The glycogen decrease following extrahepatic biliary obstruction was earlier described biochemically (11) *Edlund* (11) showed the *decrease to* be a disturbance of the glycogen storing function of the liver, being due, at least partly, to a lack of adenosine triphosphate Retentions of bile acids was thought to be the primary cause It is interesting to note that unconjugated bilirubin has later been shown to uncouple the oxidative phos*phorylation i e to decrease the production of* adenosine triphosphate (10, 31) The behaviour of conjugated bilirubin remains undefined, but both types of bilirubin are increased during extrahepatic biliary obstruc*tion (24) The connection between the men*tioned enzymatic changes and the glycogen decrease remains unclear and requires further investigation

Biliary Obstruction and Lysosomes

The increased amount of reaction product in hepatocytes and the increased numbers of positive Kupffer cells revealed by staining for acid phosphatase and beta glucuronidase activity *are* correlated with the ultrastructural finding of increased numbers of lysosomes and phagosomes *(23 25) both in hepatocytes* and in Kupffer cells Thus, there is reason to interpret the increased staining as an increase in numbers of enzyme molecules The increased numbers of lysosomes and phagosomes could be due to phagocytosis of damaged organelles however ultrastructural *findings also suggest a lysosomal digestion of* bile pigment during biliary obstruction (12) The lysosomes seem to have functions related to bile secretion also under physiological conditions This assumption gains strength from the normal orientation of these organelles alon[illegible] the bile canaliculi and from the

---

Fig 7 *a* T 8424/70 Normal human liver Strong canalicular activity, weaker sinusoidal activity mainly around portal zone at the bottom

b T 6884/69 Human liver 3 weeks' obstruction Proliferating bile ducts are positive Thick canaliculi

c Normal rat liver with canalicular activity

d Normal dog liver with positive Kupffer cells Leucine aminopeptidase reaction Pale nuclear counterstaining

× 178

fact that activities of acid phosphatase and beta-glucuronidase are higher in bile than in serum (28) Iron is known to leave the lysosomes of hepatocytes and to enter the bile (5) On the other hand, liver fractionation studies after injection of tritium labelled unconjugated bilirubin (4, 6) have not revealed any accumulation of radioactive material in the lysosomal fraction Thus, it is not known whether the conjugated bilirubin and the bile salts are secreted into the canaliculi by lysosomes

The large lumps of lysosomal enzyme reaction product of human liver localized close to biliary pigments, especially to biliary casts, appeared homogeneous in the acid phosphatase reaction and granular in the beta glucuronidase reaction In normal livers, both in man and animal, the reaction products of the two enzymes were coarsely granular in hepatocytes, they were more finely granular or diffuse and homogeneous in Kupffer cells Thus, it might be assumed that acid phosphatase activity during biliary obstruction increases mainly in Kupffer cells and beta-glucuronidase activity mainly in hepatocytes The assumption that conjugated bilirubin is deglucuronidated in hepatic tissue during biliary obstruction would be compatible with such an explanation (24)

Bile acids and unconjugated bilirubin are detergents and the former are known to be labilizers of the lysosomal membrane (33) It might be assumed that the permeability of the lysosomal membrane would be increased during biliary obstruction, resulting either in an increased inflow of substrate into lysosomes or an outflow of enzyme molecules In the former case, the product would appear more rapidly than under normal conditions, but its intracellular localization would remain unchanged In the latter case, a diffuse cytoplasmic staining would appear, provided that not all of the enzyme molecules had diffused into the incubation medium The large lumps of reaction product close to biliary pigment in the acid phosphatase reaction in livers of dogs and man could correspond to a diffuse cytoplasmic staining These lumps appear to be similar in unfixed and aceton fixed cryostat section from frozen liver tissue and in cryostat section from frozen tissue prefixed in buffered formalin with 0 22 M sucrose using the same incubation medium Assuming an enzyme leakage, a marked decrease in staining intensity would be expected in the unfixed sections More thorough histochemical investigations are needed, however, to determine the rôle of a detergent effect on the occurrence of the acid phosphatase bodies around biliary pigment

The increased staining of non specific esterase in cholestatic areas of livers in man and dogs might reflect an increase in *acid* esterase in lysosomes, but also an increase in lipase activated by retained bile salts

Biliary Obstruction and Membrane-Associated Hydrolases

All the enzymes studied—adenosine tri phosphatase, alkaline phosphatase, leucine aminopeptidase, gamma glutamyl transpeptidase—are known to show increased serum activity during extrahepatic biliary obstruction Their histochemical patterns changed markedly, partly with alternating increase and decrease in the sinusoids and canaliculi Adenosine triphosphatase exhibited largely the same reaction pattern in the three species both before and during biliary obstruction The characteristic loss of canalicular activity centrolobularly demonstrated by this reaction has been ascribed to the morphological loss of microvilli (34) The loss of canalicular leucine aminopeptidase activity in livers of man and rat could be related to loss of microvilli, but the negative canalicular areas are larger in adenosine triphosphatase staining These two enzymes probably differ in their localization In the normal human liver, the canaliculi appear much more branched and knotty in the leucine aminopeptidase than in the adenosine triphosphatase reaction

The cause of the increased serum alkaline phosphatase activity in biliary obstruction has been the subject of much debate It has recently been shown that the hepatic synthesis of this enzyme is increased in the rat during

biliary obstruction (20) *Hill & Sammons* (17) have demonstrated that two hepatic iso enzymes occur in man, one migrating to the position of the serum beta globulins and one to that of the serum gamma globulins The former mainly increases in parenchymal diseases, the latter in biliary obstruction The gamma globulin iso enzyme is found in the bile but not in the serum under normal condition It is tempting to ascribe the activity at the sinusoids to the beta globulin iso enzyme and the activity at the canaliculi to the gamma globulin iso-enzyme This possibility is supported by the fact that the dog in which the hepatic alkaline phosphatase activity is localized to canaliculi showed the largest serum increase during biliary obstruction while in man and rat the serum activity increase was of equal size and lower than that in the dog, the canalicular activity was faint (See Fig 1)

It is not known with certainty whether an increased hepatic synthesis during biliary obstruction occurs in the case of the other membrane associated enzymes mentioned 5 nucleotidase (=adenosine monophosphatase) increases in the serum in the rat, but not biochemically in the liver during biliary obstruction (20)

The serum values for gamma glutamyl transpeptidase activity in the three species varied with the histochemical patterns The serum increase was by far the highest in man where the liver activity during biliary obstruction increases in the canaliculi The smallest rise was observed in the rat, in which no canalicular activity was ever observed In this animal, the proliferating ductular epithelium showed activity In the dog liver there was activity of gamma glutamyl transpeptidase in periportal canaliculi and a moderate increase in serum activity was noticed (See Fig 1)

REFERENCES

1 *Aronsen, K F, Hagerstrand I & Norden, J G* Enzyme studies in dogs with extrahepatic biliary obstruction Scand J Gastroent *3* 355–368, 1968

2 *Aronsen, K F Hagerstrand, I & Nordén, J G* Enzyme studies in man with extrahepatic biliary obstruction Acta path microbiol. scand Sec A *80* 501–508, 1972

3 *Aronsen, K F, Hagerstrand I, Nordén, J G & Ohlsson, E G* Histochemical studies of hepatic enzymes in dogs subjected to barbiturate anaesthesia Europ Surg Res In press 1973

4 *Bernstein, L H, Ezzer, J B, Gartner, L & Arias, I M* Hepatic Intracellular Distribution of tritium labeled unconjugated and conjugated bilirubin in normal and Gunn rats J Clin Invest *45* 1194–1201, 1966

5 *Bradford W D, Elchlepp J G, Arstila, A U, Trump, B F & Kinney, T D* Iron metabolism and cell membranes. I Relation between ferritin and hemosiderin in bile and biliary excretion of lysosome contents Am J Path *56* 201–228, 1969

6 *Brown, W R, Grodsky G M & Carbone J W* Intracellular distribution of tritiated bilirubin during hepatic uptake and excretion Am J Physiol *207* 1237–1241 1965

7 *Cameron, G R & Muzaffar Hasan, S* Disturbances of structure and function in the liver as the result of biliary obstruction J Path Bact *75* 333 349, 1958

8 *Carruthers J S & Steiner, J W* Experimental extrahepatic biliary obstruction Fine structural changes of liver cell mitochondria Gastroent *42* 419–430 1962

9 *Chou, S T & Gibson, J B* A histochemical study of the bile ducts in long term biliary obstruction in the rat J Path *103* 163–175, 1971

10 *Cowger, M Igo R P & Labbe, R F* The mechanism of bilirubin toxicity studied with purified respiratory enzyme and tissue culture systems Biochem *4* 2763–2770 1965

11 *Edlund, Y* Studies on the carbohydrate metabolism and liver protection therapy in experimental extrahepatic biliary obstruction Acta Chir Scand Vol *96* Suppl 136 1948

12 *Essner, E & Novikoff, A B* Human hepatocellular pigments and lysosomes J Ultrastructure Research *3* 374–391 1960

13 *Greim, H Trulzsch D, Roboz J, Dressler, K Czygan P, Hutterer, F, Schaffner, F & Popper H* Mechanism of cholestasis 5 Bile acids in normal rat livers and in those after bile duct ligation Gastroenterology *63* 837–845 1972

14 *Greim H Trulsch D, Czygan P, Rudick, J Hutterer F Schaffner F & Popper, H* Mechanism of cholestasis 6 Bile acids in human livers with or without biliary obstruction Gastroent *63* 846–850 1972

15 *Haslewood G A D* Recent developments in our knowledge of bile salts Physiol Reviews *35* 178–196 1955

16 *Heimann, R* Factors producing liver cell necrosis in experimental obstruction of the common bile duct J Path Bact *90* 479–485, 1965
17 *Hill, P G* & *Sammons, H G* An interpretation of the elevation of serum alkaline phosphatase in disease J Clin Path *20* 654–659, 1967
18 *Hagerstrand, I* & *Nordén, J G* Enzyme studies in rats with extrahepatic biliary obstruction Acta path microbiol scand Sec A *80* 539–547, 1972
19 *Hagerstrand, I, Nordén, J G* & *Ohlsson, E G* Histochemical studies of hepatic enzymes in dogs subjected to lobar bile duct obstruction To be published
20 *Kaplan, M M* & *Righetti, A* Induction of rat liver alkaline phosphatase the mechanism of the serum elevation in bile duct obstruction J Clin Invest *49* 508–516, 1970
21 *Korb, G, Gedigk, P, Muller, R* & *Muller, V* Tierexperimentelle Untersuchungen uber die Netznekrose in der Leber Virchows Arch Abt A Path Anat *347* 357–368, 1969
22 *Lopez, M* & *Mazzanti, L* Experimental investigations on alpha naphthyl iso thiocyanate as a hyperplastic agent of the biliary ducts in the rat J Path Bact *69* 243 250 1955
23 *Orlandi, F* Electron microscopic observations on human liver during cholestasis Acta hepato-splen *9* 155–164, 1962
24 *Raia, S* Histochemical demonstration of conjugated and unconjugated bilirubin in cholestatic liver tissue Bilirubin metabolism pp 285 289 Blackwell Oxford/Edinburgh, 1967
25 *Schaffner, F* & *Popper, H* Cholestasis is the result of hypoactive hypertrophic smooth endoplasmic reticulum in the hepatocyte Lancet 2 355–359 1969
26 *Schaffner, F Bacchin, P G Hutterer, F, Scharnbeck, H H, Sarkozi, L L, Denk, H,* & *Popper, H* Mechanism of cholestasis 4 Structural and biochemical changes in the liver and serum in rats after bile duct ligation Gastroent *60* 888–897, 1971
27 *Schaffner, F* & *Javitt, N B* Morphologic changes in hamster liver during cholestasis induced by taurolithocholate Lab Invest *15* 1783 92, 1966
28 *Slater, T S* & *Griffiths, D B* Studies on the enzymic composition of rat bile following the administration of various toxic substances The biliary system A symposium Ed by W Taylor, AK 1961–1965, Oxford 1965 pp 431–438
29 *Steiner, J W, Jézéquel, A M Phillips, M J, Miyai K* & *Arakawa, K* Some aspects of the ultrastructural pathology of the liver Progress in Liver Diseases, William Heinemann, Vol II, p 314, 1965
30 *Steiner, P E* & *Martinez, J B* Effects on the rat liver of bile duct, portal vein and hepatic artery ligations Am J Path *39* 257–284 1961
31 *Zetterstrom, R* & *Ernster, L* Bilirubin, an uncoupler of oxidative phosphorylation in isolated mitochondria Nature *178* 1335–1337, 1956
32 *Van Damme, B, Fevery, J* & *Heirweigh, K P M* Altered composition of bilirubin conjugates in rat bile after obstruction of the common bile duct Experientia *26* 27–28, 1971
33 *Weissman, G* Lysosomes (Concluded) New Eng J Med *273* 1143–1149 1965
34 *Wills, E J* & *Epstein, M A* Subcellular changes in surface adenosine triphosphatase activity of human liver in extrahepatic obstructive jaundice Am J Path *49* 605–615 1966

EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits

5 Reversibility of Skeletal Fluorosis after Completed Growth

JAN ROSENQUIST

The Departments of Histology, Oral Surgery and Orthopaedic Surgery,
University of Umeå, S 901 87 Umeå, Sweden

Growing rabbits were given a fluoride supplement of 0 5 and 10 mg of fluoride per kg body weight per day during 14 weeks, that is, until they were 21 weeks of age At that time the fluoride supplement was withdrawn and the animals were followed for another 24 weeks Cortical diaphyseal bone from femur and tibia was studied by microradiography and fluorescence microscopy after fluorochrome labelling There were no differences in bone morphology between the group given 0 5 mg of fluoride and the control animals In animals given 10 mg of fluoride, excessive periosteal bone formation was seen at the lateral part of the tibial cortex This fluorotic bone was characterized by thin radiating and heavily labelled bone trabeculae and there was a considerable degree of porosity in this bone After withdrawal of the fluoride, no further signs of trabecular bone formation were seen With increasing time of observation the porosity gradually decreased The findings support the view that change from a high fluoride intake to a normal diet will result in progressive normalization of bone morphology This normalization will occur in spite of maintained fluoride contents in the bone ash

The changes in bone morphology known as chronic skeletal fluorosis have been studied in different experimental animals as well as in man (*Roholm* 1937, *Bauer* 1945, *Weatherell & Weidmann* 1959) More recently, the development of skeletal fluorosis has been studied in growing rabbits by *Brearly & Storey* (1970) and *Malcolm & Storey* (1971) Growing rabbits were supplied ad libitum with water containing fluoride In the diaphyses of femur and tibia alterations developed which were characterized by a complex pattern of excessive bone formation and bone resorption The bone formation occurred mostly periosteally and in the form of thin radiating trabeculae Endosteal resorption diminished the original prefluorotic part of the cortex Resorption cavities were found both within the original bone tissue and in the bone formed during fluoride ingestion

To the author's knowledge no morphological studies on the regression of bone fluorosis have been reported in the literature

The aim of the present investigation was to study from a morphological point of view, the *reversibility* of skeletal fluorosis after withdrawal of the fluoride supplement

The study was carried out on cortical diaphyseal bone of the femur and the tibia of rabbits Growing rabbits were given known amounts of fluoride during the growth period

At 21 weeks of age, when skeletal growth

Received 15 v 73 Accepted 15 v 73

had ceased, the fluoride supplement was withdrawn and the rabbits were followed for another 24 weeks Bone morphology was studied using fluorescence microscopy and microradiography Morphometric analyses were carried out on parts of the material

MATERIAL AND METHODS

Growing rabbits of both sexes, aged 48–52 days at the beginning of the experiment, were fed a basic diet containing 2 p p m of fluoride (for composition, see *Rosenquist* 1973) In two experimental groups this diet was supplemented with 0 5 mg (II A) and 10 mg (II B) of fluoride per kg body weight per day respectively After 14 weeks the supplement was withdrawn and the animals were followed for another 24 weeks. Throughout the experiment the animals were supplied with distilled water

The animals were killed by an overdose of sodium pentobarbital and femora and tibiae were freed from soft tissues The bones were divided with a bandsaw as shown in Fig 1

For this study diaphyseal samples from the distal

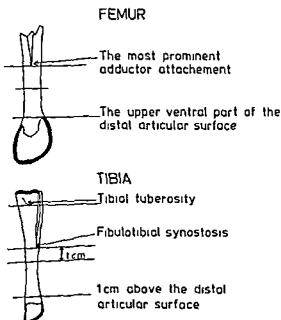


Fig 1 The bones were divided at indicated levels at right angles to their long axis The mid diaphysis of the right femur was divided into one proximal half used for histochemical purposes and one distal half used for morphological studies The right tibia was divided into a proximal piece used for histochemical purposes and a distal piece used for morphological examination Samples from the left femur and tibia were used for chemical analyses

part of the right femur and the right tibia were chosen The bone segments were dehydrated in absolute ethanol and embedded in methyl methacrylate Cross sections, about 1 mm thick, were sawn from the proximal ends of the embedded specimens and were ground down to a thickness of 80–100 μ Care was taken to insure that these sections were from corresponding parts of the respective bones A few specimens were lost during the preparation

The distal femoral epiphyseal growth zone was divided in the sagital plane to establish if longitudinal growth was completed This study was based on the fluorochrome labelling as visualized under ultraviolet light

Samples of bone were obtained at the time of withdrawal of the fluoride supplement (day 0) viz. 21 weeks of age and at 25, 33 and 45 weeks of age.

Fluorochrome labelling One week before death all animals were given a subcutaneous injection of a 2 per cent solution of 2,4 bis [N, N di(carbomethyl) aminomethyl] fluorescein (DCAF) (Nutritional Biochemicals Corp, Cleveland, Ohio, USA) (*Suzuki* & *Mathews* 1966) in a 2 per cent aqueous solution of sodium bicarbonate (pH 8 3) at a dosage of 1 ml per kg body weight This labelling will be referred to as FII Two days before death the animals were given an intramuscular injection of oxytetracycline (Pfizer) at a dosage of 12 5 mg per kg body weight (FIII) At the time of withdrawal of the fluoride supplement those animals which were followed for another 4, 12 and 24 weeks were given an injection of oxytetracycline as described above (FI)

For fluorescence microscopic examinations the specimens were mounted in Eukitt (O Kindler, Freiburg, W Germany) and examined in a Zeiss fluorescence microscope The combination of exciter filters BG 38 & BG 3 and barrier filters 47 and 65 was used

The extent of fluorochrome labelling of the endosteal and periosteal margins was scored in the following way 0 = no labelling, 1 = < ⅓ of the margin labelled, 2 = ⅓ but < ⅔ of the margin labelled 3 = ⅔ but not the entire margin labelled 4 = the entire margin labelled

Microradiography was performed using a Siemens tube (AGW 3 o, with a 1 mm² tungsten target and a 0 1 mm thick beryllium window) at 15 kV, the sections were placed in direct contact with Kodak spectroscopic plates 649 0

Morphometric analyses of the relative porosity of the bone tissue was determined by the point sampling method according to *Chalkley* (1943) ...

radiographs of one tibial cross section from each animal Areas of bone in which there were no resorption cavities (Figs 2 and 3) were selected at



Fig 2 Fluorotic area of cross sectioned tibial bone as viewed with an integrating eyepiece × 320

ıdom for the measurements The parts of the ne not occupied by mineralized tissue or re senting osteocyte lacunae were regarded as orosities'

Statistics The degree of significance was tested ith Student's t test

Determinations were done in duplicate on 5 or 6 ındomly selected samples from each group The recision of the determinations was calculated as he standard deviation of the single observation vith the formula $s_e = \sqrt{\frac{\Sigma d^2}{2n}}$, where d denotes the difference between respective pairs of samples and n the number of double analyses performed

RESULTS

The growth zones of the distal femoral epiphyses showed some activity at 21 but none at 25 weeks of age

There were no visible alterations in the bone tissue in group II A as compared with the controls

In group II B the morphological changes in tibia and femur will be described separately as there was a considerable difference between these two bones in the stimulation of new bone formation by fluoride supplement

In Table 1 the scores of the fluorochrome labelling are presented There were no differences between FII and FIII in regard to the scoring The values for FII-FIII constitute the total marginal distance labelled By reading Table 1 it should be noted that the FII-FIII-scores at day O give information corresponding to that given by FI at the other observation times viz. the new bone formation activity at the time of withdrawal of the fluoride supplement Furthermore the scores given for FI are influenced by both bone



Fig 3 Normal bone area as viewed with an integrating eyepiece × 320

TABLE 1 *Scores of the Tibial (a) and Femoral (b) Labelling*

Obs time weeks	Score	Endosteal margin labellings				Periosteal margin labellings			
		FI		FII–FIII		FI		FII–FIII	
		C	E	C	E	C	E	C	E
a) Tibial scores									
	0			0	1			0	0
	1			0	3			0	0
0 (21)	2			1	2			0	0
	3			3	0			2	0
	4			3	0			5	6
	n			7	6				
	0	0	3	0	0	0	6	0	1
	1	0	4	1	2	0	1	0	3
4 (25)	2	2	1	2	4	0	1	0	1
	3	1	0	2	2	1	0	0	3
	4	2	0	0	0	4	0	5	0
	n	5	8						
	0	1	6	0	1	0	0	0	1
	1	1	0	0	4	0	1	0	1
12 (33)	2	2	1	5	2	1	3	0	2
	3	1	0	0	0	2	1	2	1
	4	0	0	0	0	2	2	3	2
	n	5	7						
	0	0	4	0	0	0	2	0	0
	1	0	3	4	3	0	3	3	1
24 (45)	2	6	1	2	2	2	3	2	1
	3	0	0	1	2	4	0	1	4
	4	1	0	0	1	1	0	1	2
	n	7	8						

Observation times in weeks calculated from the day of withdrawal of the fluoride supplement. Actual age within brackets C = controls, E = experimental animals

formation activity and resorption of labelled bone

TIBIA

Fluorochrome Scoring

In the *control group*, the endosteal circumferential labelling extended over a large portion of the border at day O The extent of the labelling showed a tendency to decrease with increasing age The periosteal margin was almost entirely labelled except at 33 and 45 weeks of age

At all observation times the endosteal labelling accomplished at day O was less extended in the *experimental group* than in the control group FII FIII showed a weak tendency towards an increase with time after withdrawal of the fluoride supplement The periosteal margin was entirely labelled at day O A clear decrease of FI with increasing observation time was seen in most animals After 12 weeks of observation three animals, however showed a high labelling score attributable to fluorochromation at day 0

With increasing observation time the extent of FII-FIII decreased compared with day O as well as with the controls except at 24 weeks of observation

TABLE 1 *Cont*

Obs. time weeks	Score	Endosteal margin labellings FI C	FI E	FII-FIII C	FII-FIII L	Periosteal margin labellings FI C	FI E	FII-FIII C	FII-FIII E
b) Femoral scores									
0 (21)	0			0	1			1	0
	1			0	1			1	0
	2			0	4			2	0
	3			2	0			1	1
	4			1	0			1	5
	n			6	6				
4 (25)	0	0	3	0	1	0	4	0	0
	1	0	2	1	2	2	0	0	1
	2	1	2	3	2	2	1	0	1
	3	4	1	1	3	1	0	4	2
	4	0	0	0	0	0	0	1	1
	n	5	8						
12 (33)	0	0	2	1	0	2	1	0	0
	1	0	2	1	4	2	1	0	3
	2	1	1	2	1	1	1	1	2
	3	1	1	1	1	0	3	3	0
	4	3	0	0	0	0	0	1	1
	n	5	6						
(45)	0	1	5	0	0	2	0	0	0
	1	0	3	2	3	1	3	3	2
	2	3	1	2	4	0	4	0	2
	3	2	0	1	1	3	2	2	3
	4	0	0	1	1	0	0	1	2
	n	6	9						

serva ion times in weeks calculated from the day of withdrawal of the fluoride supplement Actual within brackets C = controls, B = experimental animals

rther Observations

In the *control group*, circumferential bone mellae were seen at all *observation* times xcept endosteally at day O At that time ndosteal sites of resorption were seen in some animals Signs of intense bone *formation* were not seen in the lateral part of the cortex This is in contrast to the *experimental* group (see below)

In the *experimental group* there was at day 0 excessive periosteal bone *formation* in the lateral part of the cortex with thin, radiating and heavily labelled trabeculae (Fig 4 a)

In such areas of excessive bone formation the trabecular surfaces within the bone were also extensively labelled (Fig 5) This newly formed bone was excessively rich in osteocytes After withdrawal of the fluoride supplement no signs of further marginal trabecular bone formation were seen Surface labelled trabeculae within the bone were seen less frequently with increasing observation time Endosteal resorption was a common feature even at 24 weeks of observation (Fig 6) Resorption cavities were usually present in the bone formed before fluoride ingestion but were also seen even in the fluorotic bone (Fig 4 b), they became less numerous with time

TABLE 1 *Scores of the Tibial (a) and Femoral (b) Labelling*

Obs time weeks	Score	Endosteal margin labellings				Periosteal margin labellings			
		FI		FII–FIII		FI		FII FIII	
		C	E	C	E	C	E	C	E
a) Tibial scores									
	0			0	1			0	0
	1			0	3			0	0
0 (21)	2			1	2			0	0
	3			3	0			2	0
	4			3	0			5	6
	n			7	6				
	0	0	3	0	0	0	6	0	1
	1	0	4	1	2	0	1	0	3
4 (25)	2	2	1	2	4	0	1	0	1
	3	1	0	2	2	1	0	0	3
	4	2	0	0	0	4	0	5	0
	n	5	8						
	0	1	6	0	1	0	0	0	1
	1	1	0	0	4	0	1	0	1
12 (33)	2	2	1	5	2	1	3	0	2
	3	1	0	0	0	2	1	2	1
	4	0	0	0	0	2	2	3	2
	n	5	7						
	0	0	4	0	0	0	2	0	0
	1	0	3	4	3	0	3	3	1
24 (45)	2	6	1	2	2	2	3	2	1
	3	0	0	1	2	4	0	1	4
	4	1	0	0	1	1	0	1	2
	n	7	8						

Observation times in weeks calculated from the day of withdrawal of the fluoride supplement Actual age within brackets C = controls, E = experimental animals

formation activity and resorption of labelled bone

TIBIA

Fluorochrome Scoring

In the *control group*, the endosteal circumferential labelling extended over a large portion of the border at day O The extent of the labelling showed a tendency to decrease with increasing age The periosteal margin was almost entirely labelled except at 33 and 45 weeks of age

At all observation times the endosteal labelling accomplished at day O was less extended in the *experimental group* than in the control group FII-FIII showed a weak tendency towards an increase with time after withdrawal of the fluoride supplement The periosteal margin was entirely labelled at day O A clear decrease of FI with increasing observation time was seen in most animals After 12 weeks of observation three animals, however, showed a high labelling score attributable to fluorochromation at day 0

With increasing observation time the extent of FII-FIII decreased compared with day O as well as with the controls except at 24 weeks of observation

TABLE 2. Cont.

Obs. time weeks	Score	Endosteal surface [illegible] FI C	FI E	FII FIII C	FII FIII E	Periosteal surface [illegible] FI C	FI E	FII FIII C	FII FIII E
(b) Femoral scores									
0 (21)	0			2	2			1	3
	1			0	[illegible]			[illegible]	[illegible]
	2			0	4			[illegible]	0
	3			[illegible]	[illegible]			1	1
	4			[illegible]	[illegible]			[illegible]	[illegible]
	n			[illegible]	[illegible]				
4 (25)	0	0	3	[illegible]	1	2	4	0	0
	1	0	[illegible]	1	[illegible]	[illegible]	[illegible]	1	1
	2	1	[illegible]	3	[illegible]	[illegible]	4	4	4
	3	4	[illegible]	[illegible]	3	1	[illegible]	1	2
	4	0	3		0	3	3		1
	n	[illegible]	8						
12 (33)	0	0	2	1	[illegible]	2	1	2	0
	1	0	2	[illegible]	4	2	1	0	3
	2	1	1	2	1	1	1	1	[illegible]
	3	1	1	1	1	0	3	3	0
	4	3	0	3	0	0	0	1	1
	n	5	[illegible]						
24 (45)	0	1	3	0	0	2	0	0	0
	1	0	3	2	3	1	3	3	2
	2	3	1	2	4	0	[illegible]	0	2
	3	2	0	1	1	3	2	2	3
	4	0	0	1	1	3	0	1	2
	n	6	9						

Observation times in weeks calculated from the day of withdrawal of the fluoride supplement. Actual age within brackets. C = controls, B = experimental animals.

Further Observations

In the control group, circumferential bone lamellae were seen at all observation times except endosteally at day 0. At that time endosteal sites of resorption were seen in some animals. Signs of intense bone formation were not seen in the lateral part of the cortex. This is in contrast to the experimental group (see below).

In the experimental group there was at day 0 excessive periosteal bone formation in the lateral part of the cortex with thin, radiating and heavily labelled trabeculae (Fig. 4a).

In such areas of excessive bone formation the trabecular surfaces within the bone were also extensively labelled (Fig. 5). This newly formed bone was excessively rich in osteocytes. After withdrawal of the fluoride supplement no signs of further marginal trabecular bone formation were seen. Surface labelled trabeculae within the bone were seen less frequently with increasing observation time. Endosteal resorption was a common feature even at 24 weeks of observation (Fig. 6). Resorption cavities were usually present in the bone formed before fluoride ingestion but were also seen even in the fluorotic bone (Fig. 4b), they became less numerous with time.

TABLE 1 *Scores of the Tibial (a) and Femoral (b) Labelling*

Obs time weeks	Score	Endosteal margin labellings				Periosteal margin labellings			
		FI		FII–FIII		FI		FII FIII	
		C	E	C	E	C	E	C	E
a) Tibial scores									
	0			0	1			0	0
	1			0	3			0	0
0 (21)	2			1	2			0	0
	3			3	0			2	0
	4			3	0			5	6
	n			7	6				
	0	0	3	0	0	0	6	0	1
	1	0	4	1	2	0	1	0	3
4 (25)	2	2	1	2	4	0	1	0	1
	3	1	0	2	2	1	0	0	3
	4	2	0	0	0	4	0	5	0
	n	5	8						
	0	1	6	0	1	0	0	0	1
	1	1	0	0	4	0	1	0	1
12 (33)	2	2	1	5	2	1	3	0	2
	3	1	0	0	0	2	1	2	1
	4	0	0	0	0	2	2	3	2
	n	5	7						
	0	0	4	0	0	0	2	0	0
	1	0	3	4	3	0	3	3	1
24 (45)	2	6	1	2	2	2	3	2	1
	3	0	0	1	2	4	0	1	4
	4	1	0	0	1	1	0	1	2
	n	7	8						

Observation times in weeks calculated from the day of withdrawal of the fluoride supplement Actual age within brackets. C = controls, E = experimental animals.

formation activity and resorption of labelled bone

TIBIA

Fluorochrome Scoring

In the *control group* the endosteal circumferential labelling extended over a large portion of the border at day O The extent of the labelling showed a tendency to decrease with increasing age The periosteal margin was almost entirely labelled except at 33 and 45 weeks of age

At all observation times the endosteal labelling accomplished at day O was less extended in the *experimental group* than in the control group FII-FIII showed a weak tendency towards an increase with time after withdrawal of the fluoride supplement The periosteal margin was entirely labelled at day O A clear decrease of FI with increasing observation time was seen in most animals.

With increasing observation time the extent of FII-FIII decreased compared with day O as well as with the controls except at 24 weeks of observation

TABLE 1 *Cont*

Obs time weeks	Score	Endosteal margin labellings				Periosteal margin labellings			
		FI		FII FIII		FI		FII FIII	
		C	E	C	E	C	E	C	E
b) Femoral scores									
0 (21)	0			0	1			1	0
	1			0	1			1	0
	2			0	4			2	0
	3			2	0			1	1
	4			4	0			1	5
	n			6	6				
4 (25)	0	0	3	0	1	0	4	0	0
	1	0	2	1	2	2	0	0	1
	2	1	2	3	2	2	4	0	4
	3	4	1	1	3	1	0	4	2
	4	0	0	0	0	0	0	1	1
	n	5	8						
12 (33)	0	0	2	1	0	2	1	0	0
	1	0	2	1	4	2	1	0	3
	2	1	1	2	1	1	1	1	2
	3	1	1	1	1	0	3	3	0
	4	3	0	0	0	0	0	1	1
	n	5	6						
24 (45)	0	1	5	0	0	2	0	0	0
	1	0	3	2	3	1	3	3	2
	2	3	1	2	4	0	4	0	2
	3	2	0	1	1	3	2	2	3
	4	0	0	1	1	0	0	1	2
	n	6	9						

Observation times in weeks calculated from the day of withdrawal of the fluoride supplement Actual age within brackets C = controls B = experimental animals

Further Observations

In the control group, circumferential bone lamellae were seen at all observation times except endosteally at day O At that time endosteal sites of resorption were seen in some animals Signs of intense bone formation were not seen in the lateral part of the cortex This is in contrast to the experimental group (see below)

In the *experimental group* there was at day 0 excessive periosteal bone formation in the lateral part of the cortex with thin, radiating and heavily labelled trabeculae (Fig 4 a)

In such areas of excessive bone formation the trabecular surfaces within the bone were also extensively labelled (Fig 5) This newly formed bone was excessively rich in osteocytes After withdrawal of the fluoride supplement no signs of further marginal trabecular bone formation were seen Surface labelled trabeculae within the bone were seen less frequently with increasing observation time Endosteal resorption was a common feature even at 24 weeks of observation (Fig 6) Resorption cavities were usually present in the bone formed before fluoride ingestion but were also seen even in the fluorotic bone (Fig 4 b), they became less numerous with time

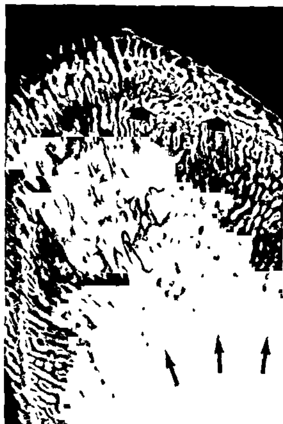


Fig 4 a Fluorescence micrograph from the lateral part of a tibial cross section of a fluoride supplemented (10 mg/kg/day) rabbit at day 0 (14 weeks on fluoride supplement) Excessive periosteal bone formation with radiating trabeculae is seen The central part of the newly formed bone is occupied by a large area mainly showing active resorption White arrows indicate the approximate borderline between fluorotic and prefluorotic bone Black arrows indicate the bone area labelled by FII All bone peripherally to this border has been formed within the last week before death × 44



of resorption (white arrows) and surfaces labelled by FII and FIII (black arrows) × 100

The bone formed during fluoride ingestion became increasingly more normal in appearence (Fig 7 and 8) At 12 and 24 weeks of observation even entire periosteal circumferential lamellae were seen in some cases

FEMUR

Fluorochrome Scoring

In the *controls*, the labelling at the endosteal margin accomplished at day 0 was fairly extensive in most animals at all observation times FII-FIII decreased slightly with in-



Fig 5 Fluorescence micrograph of a tibial cross section from an area identical to that depicted in Fig 4 In this section fluorotic bone constitutes the total thickness of the cortex Most bone surfaces are labelled by FII and/or FIII × 100



Fig 6 Micrograph of a tibial cross section of a fluoride supplemented (10 mg/kg/day) rabbit at 24 weeks of observation Arrows indicate site of resorption (white arrows) and formation of not fully mineralized lamellar bone (black arrows) × 44

creasing age Roughly ⅓ of the periosteal border was labelled at all observation times

In the *experimental group*, labelling at the endosteal margin attributable to fluoro formation at day 0 was less extensive than in the controls throughout the experiment FII FIII was seen to the same extent as in the controls except at day 0 Periosteally, almost the entire border was labelled at day 0 and thus far more extensively than in the controls At the other observation times, the extent of FI labelling was similar to that in the controls The periosteal extension of FII FIII was almost the same at all observation times at day 0

Further Observations

No signs of endosteal resorption were seen in the controls whereas it was a common feature in the ventral endosteal bone areas in the experimental group This resorption became less pronounced with increasing time of observation and resulted in a highly irregular border Within the ventral part of the cortex labelled osteones and resorption cavities could be seen especially at the shorter observation times

Well developed circumferential lamellae

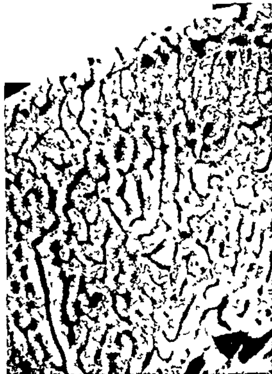


Fig 7 Micrograph of the lateral part of a tibial cross section of a fluoride supplemented (10 mg/kg/day) rabbit after 4 weeks of observation Considerable porosity and richness of osteocytes is seen Almost the entire cortex consists of fluorotic bone The porosities near the periosteal margin are sites of bone formation (black arrows) × 70

were seen at 25 weeks of age in the control group but were not found in the experimental group until 24 weeks of observation

The microradiographs showed no marked difference between control and experimental animals with respect to degree of mineralization

MORPHOMETRIC ANALYSIS

As shown in Table 2, the area of porosities of fluorotic bone decreased after withdrawal of the fluoride supplement The most rapid decrease occurred between day 0 and 4 weeks of observation However, even at 24 weeks after withdrawal of the fluoride supplement porosities were present but occupied a fairly small area



Fig 8 Microradiograph of a tibial cross section of a fluoride supplemented (10 mg/kg/day) rabbit after 24 weeks of observation The approximate border between the fluorotic and prefluorotic bone is indicated by black arrows. Peripherally to the fluorotic bone a circumferential lamella is develop ing (white arrows) Lamellar bone is also seen endosteally × 44

DISCUSSION

The lack of morphological alterations in the low fluoride group (0 5 mg of F /kg/day, corresponding to an intake of water contain ing roughly 5 ppm of F) are in agreement with findings at necropsies of man in areas with 1–4 ppm of fluoride in the drinking water (*Geever et al* 1958) In this group, a fluoride content of 0 07 per cent was found in the bone ash at completed growth (*Rosenquist* 1973) The present findings indicate that about 5 ppm of fluoride in the water does not affect normal bone development in the rabbit In contrast, the intake of 10 mg/kg/day during growth giving a content of 0 7 per cent, resulted in development of marked bone alterations These alterations henceforth called bone fluorosis, were characterized by excessive periosteal bone formation and bone resorption as well as pronounced remodelling The newly formed periosteal bone was further characterized by extreme porosity This is an agreement with observations in rabbits by *Malcolm* & *Storey* (1971) The periosteal, pathologically altered bone, formed during fluoride ingestion will be referred to as fluorotic bone

One puzzling but constant finding was the localization of fluorotic bone to the lateral part of the periosteal circumference of the tibia (cf *Malcolm* & *Storey* 1971) Large individual variations between animals suggest that the sensitivity to fluoride way vary

In accordance with previous observations (*Malcolm* & *Storey* 1971) the femur showed far less bone fluorosis than the tibia at completed growth This is remarkable as the fluoride content of the bone ash was equal (*Rosenquist* 1973) It has been observed that the content and composition of the acid glycosaminoglycans show minor but significant differences in cortical bone between femur and tibia, as was true of the concentrations of hydroxyproline (*Lemperg* & *Rosenquist* 1973) However, these findings can hardly explain the excessive tibial bone formation observed in certain localized areas

TABLE 2 *Relative Porosity of Tibial Bone Expressed as per cent Non mineralized Areas per Surface Unit In the Experimental Group Periosteal Trabecular Areas and in the Control Group Compact Bone was Studied*

Obs. time	0 (21)	4 (25)	12 (33)	24 (45)
Group	n	n	n	n
Control	5 3 6 ± 1 1***	5 3 4 ± 1 2***	5 3 5 ± 1 0**	5 3 5 ± 1 1**
Exptl	6 30 0 ± 3 5	6 11 6 ± 2 2xxx	6 7 0 ± 1 3xx	6 6 4 ± 1 3

The precision of the determinations s_e = 1 0 per cent
Observation times calculated in weeks after withdrawal of fluoride supplement Age within brackets
M ± S D The statistical significance of differences between the groups is indicated by (*) and between different observation times in the experimental group by (x)
x, * 0 05 > p > 0 01 xx ** 0 01 > p > 0 001
xxx *** 0 001 > p

Furthermore, no differences between the organic composition of normal and fluorotic bone were found. The possible role of vasculatory factors in the restriction of fluorotic bone formation to certain bone areas only is at present an open question and remains to be elucidated. As bone fluorosis was most pronounced in the tibia at completed growth the discussion of the reversibility after withdrawal of the fluoride supplement will be limited to this bone. There was a clear progressive normalization of the bone structure after withdrawal of the fluoride supplement.

Scoring of surfaces labelled with different fluorochromes (*Suzuki & Mathews* 1966) gave a semi quantitative estimate of the sequence of bone formation and resorption dynamics during the period of normalization. The endosteal resorption active at the time of withdrawal decreased and was replaced by bone formation which resulted in circumferential bone lamellae.

The periosteal growth of fluorotic trabecular bone ceased. The fluorotic bone was partly resorbed and gradually outlined by a circumferential bone lamella, although a normalization period of 24 weeks was not sufficient to completely extinguish the picture of bone fluorosis.

In addition the porosity of the fluorotic bone as determined by morphometry, decreased significantly during the first 12 weeks of observation.

The obvious normalization appeared to be a direct consequence of the withdrawal of the fluoride supplement. It cannot be entirely excluded that ageing too might have been a factor in this normalization process. However, the age related changes in the control group were small and consisted mainly of a slight decrease in bone formation. Thus, ageing can hardly explain the marked regression of the extensive bone alterations. Moreover, in the study by *Malcolm & Storey* (1971), continued periosteal, trabecular bone formation was reported to occur between 100 and 200 days of observation in fluoride treated growing rabbits of about the same age as those used in this study.

It should be pointed out that the morphological normalization occurred in spite of practically unchanged fluoride contents in the bone ash (0.76 per cent at day 0 and 0.72 after 24 weeks) (*Rosenquist* 1973). Although it is difficult to explain, the noted difference between femur and tibia is remarkable as the fluoride concentration was practically equal. This observation strongly suggests that the effects of fluoride are primarly directed towards osteogenic cells and that these effects are not exerted once this ion is firmly bound to the bone minerals. This assumption is further supported by the findings that, although the fluoride contents of cortical bone ash were similar, there were large variations in bone morphology in different animals. Thus fluoride concentrations in the single bone give no information on the degree of bone fluorosis unless other criteria for the existence of bone fluorosis have been provided. The interrelation between local fluoride concentration and morphological changes is presently being studied in microdissected samples from distinctly altered bone areas.

Resorption, which is a common feature in bone fluorosis, has been claimed to be a result of secondary hyperparathyroidism (*Faccini & Care* 1965, *Faccini* 1967). This, in turn was thought to be due to an increased resistance to resorption of the fluoride containing bone. In the present study, resorption was seen mainly in areas where bone formation occurred simultaneously. It was also seen in the newly formed fluorotic bone. Resorption would thus seem to be connected with local demands and a study on the parathyroids from the same animals did not reveal any signs of increased glandular activity (*Rosenquist & Boquist* 1973).

This morphological study in rabbits on the reversibility of bone fluorosis after attained skeletal maturity supports the view that change from a high fluoride intake to a normal diet will result in progressive normalization of bone morphology. This normalization will occur in spite of maintained fluoride contents in the bone ash.

This investigation has been supported by grants from *Stiftelsen Sigurd och Elsa Goljes Minne*

REFERENCES

Bauer, W M Experimental chronic fluoride intoxication Amer J Orthodont *31* 700–719, 1945

Brearley, L J & *Storey E* Osteofluorosis in the rabbit macroscopic and radiographic changes Pathology 2 231–247, 1970

Chalkley, H W Method for the quantitative morphologic analysis of tissues J nat Cancer Inst *4* 47–53, 1943

Faccini, J M Inhibition of bone resorption in the rabbit by fluoride Nature *214* 1269 1271, 1967

Faccini, J M & *Care, A D* Effect of sodium fluoride on ultrastructure of the parathyroid glands of the sheep Nature *207* 1399–1401, 1965

Geever, E F, Leone, N C, Geiser, P & *Liebermann, J E* Pathological studies in man after prolonged ingestion of fluoride in drinking water Publ Hlth Rep (Wash) *73* 721–731, 1958

Lemperg, R & *Rosenquist, J* Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits III Concentration of acid glycosaminoglycans and hydroxyproline in cortical bone To be published 1973

Malcolm, A S & *Storey, E* Osteofluorosis in the rabbit micro-radiographic studies Pathology *3* 39–51, 1971

Roholm, K Fluoride intoxication A clinical hygienic study with a review of the literature and some experimental investigations H K Lewis, London 1937

Rosenquist, J Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits. 1 Concentration of fluoride in cortical bone Acta path microbiol scand Sect A *81* 630–636, 1973

Rosenquist, J & *Boquist, L* Effects of supply and withdrawal of fluoride Experimental studies on growing and adult rabbits. 2 Parathyroid morphology and function Acta path microbiol scand Sect A *81* 637–644, 1973

Suzuki, H K & *Mathews, A* Two colour fluorescent labelling of mineralizing tissues with tetracycline and 2,4 bis [N, N-di (carbomethyl) aminomethyl] fluorescein Stain Technol *41* 57 60 1966

Weatherell, J A & *Weidmann, S M* The skeletal changes of chronic experimental fluorosis J Path Bact *78* 233–241, 1959

Acta path microbiol scand Section A *81*, 761-769, 1973

MEASLES AND ITS RELATIONSHIP TO GIANT CELL PNEUMONIA (*HECHT* PNEUMONIA)

KJELL HARAM and KNUT JACOBSEN

The Gade Institute, Department of Pathology, University of Bergen, Norway
(Head Professor, dr med Flora Hartveit)

In an autopsy series 12 out of 152 cases of pneumonia occurring in patients under 20 years of age were found to be *Hecht* (giant cell) pneumonia (8 per cent) Histological examination of 400 pneumonias from patients over 20 years of age showed no such case Six of the cases were considered to be due to measles on the basis of eosinophilic inclusion bodies in the cytoplasm and nuclei of the giant cells, *Warthin Finkeldey* cells in the lymphoid system and on clinical data Only one had a typical exanthema All cases with measles were between 2 and 11 years of age. In the remaining 6 cases there was no morphological or clinical evidence of measles All, except one, were below 1 year of age The role of depressed immunological function in the pathogenesis of *Hecht* pneumonia is discussed In the group caused by measles the depressed immunological function may partly be iatrogenic due to immunospressive treatment of leukaemic children The other group of cases consisted of children with inborn defects some of which showed morphological signs of immunodeficiency

From the end of the 19th century a number of authors have drawn attention to the peculiar histological changes in the lungs of children who have died from measles, namely, a pneumonia dominated by multinucleated syncytical giant cells *Kromayer* (26) regarded this type of pneumonia as diagnostic of measles, and *Hecht* (18) found a clear relationship between giant cell pneumonia and measles

From the early 1930 s it has been known that diagnostic *Warthin-Finkeldey* giant cells may be found in the lymphoid system in the prodromal stage of measles (15, 46) This giant cell differs in some morphological aspects from that found in the lungs

Received 26 ii 73 Accepted 11 v 73
Requests for reprints should be addressed to Dr Kjell Haram, Haukeland Sykehus, 5000, Bergen Norway

In 1953 *Enders et al* (13) reported the isolation of measles virus from the lungs and bronchial tissues of 3 leukaemic children with so-called Hecht pneumonia None of them had a rash, but they had been exposed to measles Thus, apparently measles can occur without rash One of us has reported a similar case in a child without leukaemia, stressing the importance of the cytopathological findings for the correct diagnosis of the disease (22)

The present work was planned to investigate (i) the frequency of *Hecht* pneumonia in an autopsy series , (ii) its relationship to measles and (iii) the circumstances under which this lung reaction may occur

MATERIAL AND METHODS

From January 1st, 1959 to June 30th, 1971, 10 204 autopsies were performed at this Institute Pneu

TABLE 1 *Relationship between Age and Incidence of Giant Cell Pneumonia in Autopsy Series*

Age	No of autopsies	Pneumonia	Per cent of autopsies	No of giant cell pneumonia	Per cent of giant cell pneumonia*
1 week – 1 year	267	73	27,34	4	5,49
1 year – 4 years	137	42	30,65	5	11,90
5 years – 9 years	66	14	21 21	2	14,29
10 years – 14 years	113	8	20,35	1	12 50
15 years – 19 years		15		0	0
Total	593	152	25,63	12	7,89

* Percentage given in relation to the total number of pneumonias

monia was recorded in 1 763 cases Of these 1,611 were in the age group above 20 years, while 152 were below this but more than 1 week old Clinical details were available from the case records.

Sections from the lungs and lymphoid tissue from the latter 152 cases, and available lung sec tions from 400 cases from the group above 20 years, were examined histologically The material for histology had been fixed in formalin, paraffin embedded and stained with haematoxylin and eosin (H E) Additional sections were prepared if necessary In cases showing giant cells sections were stained with *Mayer's* Phloxine—Tartrazine for the demonstration of inclusion bodies.

RESULTS

The series was found to contain 12 cases of *Hecht* pneumonia All were in the group under 20 years The relationship between age and the incidence of *Hecht* pneumonia in this age group is given in Table 1 which shows that such cases accounted for about 8 per cent of the total number of cases of pneumonia, the percentage incidence being similar between 1 14 years

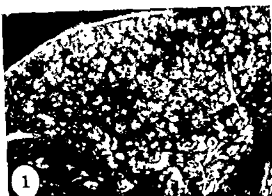
Of these 12 cases, eleven had been admitted to the Childrens' Hospital, University of Bergen, before death One, case 2, died at home

The cases were numbered in chronological order Details of age and sex are given in Table 2 There were 7 girls and 5 boys No relationship between sex and age at death was found

TABLE 2 *Twelve Cases of Giant Cell Pneumonia with Some Morphological Data of Importance for the Aetiological Diagnosis*

Case no	Year of death	Sex	Age in year	Inclusions in the giant cells of the lung		W F cells$ in lymphatic tissue	Metaplasia of the bronchus
				Nucleus	Cytoplasma		
1	1959	♀	7/12	—*	+ †	—	—
2	1959	♀	8 5/12	+	+	+	—
3	1965	♂	6/12	—	—	0§	—
4	1966	♂	8/12	—	+	0	+
5	1968	♀	11 2/12	+	+	—	+
6	1968	♂	3 5/12	—		—	+
7	1968	♀	3 11/12	+	+	+	+
8	1970	♀	3/12	—	+		+
9	1970	♀	3 8/12	—	+	—	+
10	1971	♀	6 5/12	—	—	—	+
11	1971	♂	1 10/12	+	+	+	—
12	1971	♂	4 5/12	+	+	+	+

* Absent
† Present
§ Not available
$ Warthin Finkeldey



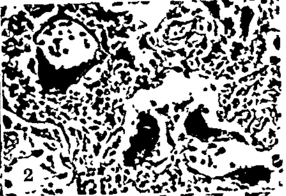






Fig 1 Cut surface of the lung in case 5 with multiple infiltrates giving a miliary appearance

Fig 3 A giant cell from the same case as in Fig 2 with intranuclear and intracytoplasmatic inclusion bodies (arrows) (H E × 850)

Fig 5 A lymph node from case 1 with marked cellular depletion, especially in the paracortical area (H E × 35)

Fig 2 Giant cell pneumonia with multinucleated giant cells in the alveoli and mononuclear cell infiltrates in the interstitium (case 7) (H E × 210)

Fig 4 *Warthin Finkeldey* cell in a lymph node from case 7 (H E × 850)

Fig 6 Thymus from case 7 with a medulla composed chiefly of stromal and epithelial cells without *Hassall's* corpuscles, and with no cortical differentiation (H E × 85)

Autopsy Findings

Macroscopically the *lungs* showed considerable variation Generally they were enlarged On section the surface was in some cases homogeneous with a firm consistency and a deep red to reddish blue colour In other cases there were palpable infiltrates These could be from a few millimeters to one centimeter in diameter, and varied from grey to greyish yellow in colour, giving the surface a miliary appearance (Fig 1)

Histologically the infiltrates were related to bronchitic and bronchiolitic changes Squamous metaplasia of the bronchial epithelium was frequent In addition to bronchial changes the majority had interstitial pneumonia with mononuclear cell infiltrates Two cases showed bronchopneumonia

The dominating microscopical feature was, however, the multinucleated giant cells in the bronchioli and alveoli Some were lying free while others adhered to the walls On low power examination they appeared to have a focal arrangement with the greatest concentration around the bronchi Giant cells were not seen in non infiltrated areas The absolute number varied, but they usually dominated the histological picture in infiltrates (Fig 2) The form and quantity of the cytoplasm in the giant cells also varied The cells were round to spherical, or halfmoon shaped Many had irregular nucleus free cytoplasmatic offshoots. The nuclei, which were mostly located centrally in numbers from 15 or 20 up to 100, were about 5 μ in diameter Their shape was round to spherical and the majority were hyperchromatic, but relatively chromatin deficient nuclei could occur in the same cell Sometimes the chromatin of the nucleus was pushed out to the periphery These cells often contained eosinophilic inclusion bodies In our material all cells with intranuclear inclusions had also similar intracytoplasmatic bodies (Fig 3) Some cases had intracytoplasmatic inclusion bodies only

Warthin Finkeldey cells were found in the *lymphatic tissue* in 4 cases These cells were somewhat smaller than those found in the lungs and did not contain inclusion bodies (Fig 4) In addition to *Warthin Finkeldey* cells, many of the lymph nodes showed a high degree of reticulum cell hyperplasia and lymphocyte depletion in the paracortical tissue (cases 1, 2 6 7, 8 9 10, 11) In addition in some of these cases there were relatively few lymphocytes and germinal centres in the primary cortex (Fig 5) The lymph nodes in case 12 were partly necrotic with almost complete cellular depletion The lymph nodes in case 3 were not available for study, in case 4 they were absent, and in case 5 they were apparently normal

TABLE 3 *Clinical Findings in 12 Cases of Hecht Pneumonia*

Case no	Measles		Coexisting disease	Duration of respiratory symptoms†	Lung X ray infiltrates
	Rash	Exposition			
1	—	—	—	6 days	+
2	+	+	—	3 days	ND§
3	—	—	Heart disease and coloboma	2 months	+
4	—	—	Swiss type of hypogammaglobulinaemia	2 months	+$
5	—	+	—	5 days	+$
6	—	—	Congenital thrombocytopenia	—	—
7	—	+	Leukaemia	14 days	+
8	—	—	Multiple malformationes Hanhart like syndrome	5 days	ND
9	—	—	Leukaemia	3 weeks	ND
10	+	+	—	17 days	+
11		+	Leukaemia	8 days	+
12	+*	—	Leukaemia	6 weeks	+

* Rubella like
† All cases but case 6 had respiratory symptoms
§ Not done
$ Complicated by pneumomediastinum

TABLE 4 *Haematological Investigations in Twelve Cases of Giant Cell Pneumonia Obtained During the Last Month of Life*

Case	Hb G per cent	Leucocytes	Lymphocytes in per cent of leuko	Thrombocytes	Gammaglob G per cent
1	13,4–14,2	4800–11800	75–58	0*	0
2	0	0	0	0	0
3	11 – 9,7	12200–16900	55–59	0	0
4	13,6–13,7	4200–11200	30–6	0	1,0
5	14,1	11500	24	0	0
6	10,5– 6,3	6500–2100	45–39	32000–8000	0,5–0,7
7	11,6	2200	10	12600	0
8	11,7–10	12100	62	0	0
9	6,8– 5,7	400–1000	0	20000–2000	0
10	11,7–10,7	24800–20700	11–13	0	1,8
11	12,1–10,1	8800–17600	40–5	23400–52000	0,7
12	14,6	8300	4	14000	0,3

* Not investigated

The *thymus in case 4* was hypoplastic with lack of lymphoid cells, no *Hassall's* corpuscles and no corticomedullary differentiation A similar thymus was seen in case 7 (Fig 6) The thymus in case 6 differed from these in having a few calcified *Hassall's* corpuscles. In cases 1,8 and 11 the thymus was normal, and in the rest of the cases there was no material available

Clinical Findings

The main clinical data are shown in Table 3, and haematological data on the 12 cases are given in Table 4 A history of exposition to measles was obtained in five cases, and two of these showed a measles like rash of a few hours (case 2) and 6 days (case 10) duration, respectively Six weeks before death, one patient (case 12), had a rash like that of rubella.

Eight of the cases had a coexisting disease Four had leukaemia, and their treatment during the last month of life is given in Table 5 In four cases congenital disorders had been diagnosed In cases 1,2 and 10 there was no history of ill health before the final disease

Case 5, an 11 year-old boy, eldest of five siblings, had respiratory symtoms, anorexia and weight loss three months before death His 6 year-old brother had the same symptoms at the same time. About 14 days before the death of case 5, all the siblings were exposed to measles and three of them developed uncomplicated measles

The day case 5 died, his 6 year-old brother was admitted to hospital with coughing, marked dyspnoea and cyanosis. X ray of the chest showed findings similar to those in his brother He had a sparse exanthema on both flanks (Hb was 14 g per cent, leucocytes 8400 mm³ with 32 per cent lymphocytes, γ globulin 1 7 g per cent) He was treated with antibiotics and given 5 ml of 12,5 per cent γ globulin He was critically ill at first but improvement was rapid so he could be discharged 11 days later Six months later he developed an encephalitis, possibly subacute sclerosing panencephalitis, but the antibody titre against measles was not high, and he is still alive, but mentally retarded

TABLE 5 *Treatment of Four Leukaemic Children During Their Last Month of Life*

Case	Predmson	Metho trexate	L-aspar ginase	6 mercapto purin	Cyclo-phospha mide	Blood transfusions	Anti-biotics
7	+	+	—	—	+	—	+
9	+	—	+	+	—	+	+
11	+	+	—	—	—	—	—
12	+	+	+	—	—	+	—

DISCUSSION

The ratio of *Hecht* pneumonia to other pneumonia in our material (8 per cent) is similar to that *Hecht* found in the part of his material which he examined under similar conditions (18). The present investigation confirms *Hecht's* observation that giant cell pneumonia is a disease of children, and also that measles is an important aetiological factor. *Hecht* pneumonia in adults has to be regarded as a rarity (31).

The histological diagnosis is based on the finding of characteristic multinuclear giant cells in the lungs (18, 23, 38). The demonstration of eosinophilic, intranuclear and intracytoplasmatic inclusion bodies in the giant cells is of great importance. Such cells in the peripheral respiratory tract in man appear to occur only with measles virus, and the inclusions contain specific virus antigens. However, the related canine distemper virus may induce similar changes in animals (1, 8, 9, 12, 14, 23).

The diagnosis of measles in cases 2, 5, 7, 11 and 12 could thus be made on the basis of the histological findings in the lungs alone. *Warthin-Finkeldey* cells, moreover, were found in lymphoid tissue in cases 2, 7, 11 and 12. The diagnosis was suspected clinically in case 2 only, but retrospectively it was stated that all these cases had been exposed to measles at the time of their last illness.

Lack of inclusion bodies does not appear to exclude measles. Case 10 had clinical measles but no inclusion bodies in the giant cells. *Intracytoplasmatic eosinophilic inclusion bodies* alone have also been reported in a definite case of measles (40).

Our 6 cases with no cytopathological or clinical evidence of measles were, with one exception (case 9), much younger, between 3 and 8 months, than those with measles. They were probably protected by maternal antibodies, and measles was therefore unlikely. While both eosinophilic intracytoplasmatic and intranuclear inclusion bodies do not seem to have been reported in such young infants (2, 38), *Adams et al.* (2) have described giant cell pneumonia with only intracytoplasmatic inclusion bodies in mainly premature infants below 6 months of age. The respiratory syncytical virus has been suspected as the aetiological agent (3, 19, 21, 41). Herpes virus does not seem to produce *multinuclear* giant cells in the respiratory tract, but the cytomegalovirus can form such cells with eosinophilic intranuclear and basophilic intracytoplasmatic inclusion bodies in other organs (5, 6, 28, 36). Parallel histological and virological studies are needed to clarify the situation.

Although *Hecht* (18) produced giant cell pneumonia in rabbits with intratracheal injections of inorganic material, his human material showed a clear relationship to measles. Vitamin A deficiency as a cause of *Hecht* pneumonia is also unlikely (10). Further, metaplasia of the bronchial epithelium, said to be of diagnostic value in measles, is seen in many other virus infections and has to be regarded as a non-specific phenomenon (4, 23, 25).

While measles has still a very high mortality rate in under-developed countries, the death rate has dropped drastically since the beginning of this century in countries with a high living standard (30, 35, 43, 47). Accurate assessment, however, requires an increased awareness of *Hecht* pneumonia and its implications.

While *Hecht's* cases were debilitated, our series contained a high percentage of treated leukaemic children and infants with congenital defects. These observations suggest that immunodeficiency may play an important role in the pathogenesis of *Hecht* pneumonia. Three of four leukaemic children suffered from giant cell pneumonia caused by measles (cases 7, 11, 12), and in one (case 9) the virus was unknown. As patients with acute leukaemia have normal humoral and cellular immunity (16, 33), the immunosuppressive treatment may have been the main cause of death (11, 12, 33, 45, 48). All our cases occurred at a time of intensified antileukaemic treatment in the Childrens Hospital (44). *Mitus et al.* (34) described four patients with leu

kaemia who developed measles while under antileukaemic treatment

The histology of the thymus in case 7 was indicative of a congenital thymus defect with resultant immunoinsufficiency The lymph nodes also showed paracortical cellular depletion The latter was, however, found in the majority of our 12 cases and may be caused by the virus, therapeutics, or the basic disease Cases 4 and 6 also showed histological abnormality of the thymus, which may explain their susceptibility to infection, virus disease in particular Combined failure, as in case 4, a Swiss type of hypogammaglobulinaemia, usually leads to early death due to multiple infections (27) The thymus in case 6 contained two calcified bodies, probably *Hassall's* corpuscles, but otherwise the structure was similar to that in case 4 This may indicate that the abnormal thymic structure was acquired Case 6 has been published as congenital hypoplastic anaemia (20)

In the remaining cases there was an unexpected reaction to measles Case 2 died at home after having had symptoms for three days and a rash for less than twelve hours If the exanthema is regarded as an index of cellular immunity, its short duration would indicate such a failure (7, 42) Case 5 died of fulminant giant cell pneumonia without exanthema following a debilitating respiratory disease The lymph nodes were normal but the thymus was not examined His brother had a similar disorder but survived Case 10 had a typical measles rash, with a normal humoral reaction according to the antibody titre against measles As the lungs showed bronchopneumonia as well as *Hecht* pneumonia death was probably due to a concurrent bacterial infection rather than immunological deficiency

The clinical diagnosis of *Hecht's* pneumonia is difficult. It is important to be aware of the condition in cases with acute or subacute respiratory disease often with pronounced dyspnoea and cyanosis If possible, serological and virological examinations should be carried out A valuable diagnostic feature in cases of measles is the demonstration of multinuclear giant cells from the oral mucous membrane (29) Radiographically one can find miliary, and sometimes more massive, condensates scattered in the lungs and a few patients, like some of our own, may develop mediastinal and subcutaneous emphysema (24, 37, 39)

At high dosage γ globulin may inhibit the viraemia in measles and thereby hinder the dissemination of the disease The observation that patients with *Bruton's* hypogammaglobulinaemia do not contract measles after reinfection may indicate that even small amounts of specific antibody can be of value (17) The relatively small doses of commercial γ globulin given to the brother of case 5 in this series who survived, may thus have been important

REFERENCES

1 *Adams, J M & Imagawa, D T* The relationship of canine distemper to human respiratory disease Pediat Clin N Amer *4* 193–201, 1957
2 *Adams J M, Imagawa, D T, Miye Yoshimori, B A & Huntington, R W* Giant cell pneumonia Pediatric *18* 888–898, 1956
3 *Adams, J M, Imagawa, D T & Zike, K* Relationship of pneumonitis in infants to respiratory syncytical virus J Lancet *81* 502 506 1961
4 *Aherne W, Bird T, Court, S D M, Gardner P S & McQuillin, J* Pathological changes in virus infections of the lower respiratory tract in children J clin Path *23* 7 18, 1970
5 *Altshuler, G & McAdams J* Cytomegalic inclusion disease of a nineteen week fetus Amer J Obstet Gynec *111* 295 298 1971
6 *Anderson W A D* Pathology 5 ed vol 2 C V Mosby Company Saint Louis 1966 p 914–915
7 *Burnet F M* Measles as an index of immunological function Lancet *II* 610–613 1968
8 *Carlstrom G* Appearance in children's sera of substances capable of neutralizing canine distemper virus Acta paediat Scand *45* 180–188, 1956
9 *Carlstrom G* Neutralization of canine distemper virus by serum of patients convalescent from measles Lancet *II* 344, 1957
10 *Chou n B* Giant cell pneumonia of infancy

as a manifestation of vitamin A deficiency Amer J Dis Child 57 489-505, 1939

11 *Cooper, M D & Eller, E M* Developmental suppression of germinal center and IgG production by predinsolone and 6 Mercaptopurine In L Fiore Donati and M G Hanna (eds) Lymphatic tissue and germinal centers in immune response Adv in Exp Med and Biol, vol 5, Plenum Press New York 1969 p 277-284

12 *Davis, B D* Microbiology Hoeber Med Division 2 ed Harper & Row New York 1970 p 1356-1363

13 *Enders, J F, McCarthy, K, Mitus, A & Cheatham, W J* Isolation of measles virus at autopsy in cases of giant-cell pneumonia without rash New Engl J Med *261* 875 881 1959

14 *Fenner, F J & White, D O* Medical Virology Academic Press, New York and London 1970 p 322

15 *Finkeldey, W* Ueber Riesenzellbefunde in den Gaumenmandeln zugleich ein Beitrag zur Histopathologie der Mandelveranderungen im Maserninkubationsstadium Virchows Arch path Anat *281* 323-329, 1931

16 *Good, R A & Finstad, J* The association of lymphoid malignancy and immunologic functions. Zarafonetis C J D (ed) Proceedings of International Conference on Leukemia Lymphoma. Lea & Febiger Philadelphia pp 175-197 1968

17 *Good, R A & Zak S J* Disturbances in gamma globulin synthesis as Experiments of Nature Pediatrics *18* 109 149 1956

18 *Hecht, V* Die Riesenzellen Pneumonie im Kindesalter Beitr path Anat *48* 263-310 1910

19 *Hokel A Parker L, Patterson W H White L L R Thompson K M & Tobin J O H* The isolation of respiratory virus from children with acute respiratory disease Lancet *I* 295-298 1963

20 *Høyeraal H M Lamvik J & Moe P J* Congenital hypoplastic thrombocytopenia and cerebral malformations in two brothers Acta Pædiat Scand *59* 185-191 1970

21 *Jacobs J W Peacock D B Corner B D, Caul E O & Clarke S K R* Respiratory syncytical and other viruses associated with respiratory disease in infants Lancet *I* 871-876, 1971

22 *Jacobsen K* Proceedings of pediatric societies. Acta Pædiat scand *58* 208-212 1969

23 *Janigan D T Giant cell pneumonia and* measles An analytical review Canada M A J *85* 741-749 1961

24 *Jonsson, A S* Mediastinal emphysema and mucocutaneous syndrome Lancet *I* 208-209, 1969

25 *Kaufmann, E* Spezielle Patholigische Anatomie 11 und 12 Auflage, II Band 3 Teil Walter de Gruyter & Co, Berlin 1960 p 1518

26 *Kromayer, E* Ueber die sogenannte Katarrhal Pneumonie nach Masern und Keuchhusten Virchows Arch path Anat *117* 452-468 1889

27 *Lamvik, J & Moe, P J* Thymic dysplasia with immunological deficiency Acta path microbiol scand *76* 349-360 1969

28 Leading article Disease caused by cytomegalo viruses Brit med J *I* 72 73 1968

29 *Lightwood, R & Nolan, R* Epithelial giant cells in measles as an aid in diagnosis. J Pediat 77 59-64, 1970

30 *Mayer, J* Measles and the state of nutrition. Postgrad Med *45* 253 254, 1969

31 *McConnel, M* Giant cell pneumonia in an adult Brit med J 2 288 1961

32 *Meadow, S R, Weller, R O & Archibald R W R* Fatal systemic measles in a child receiving cyclophosphamide for nephrotic syndrome Lancet *II* 876-878, 1969

33 *Miller, D G* Patterns of immunological deficiency in lymphomas and leukemias Ann. intern Med *57* 703-716 1962

34 *Mitus, A, Enders J F, Craig J M & Holloway A* Persistence of measles virus and depression of antibody formation in patients with giant cell pneumonia after measles. New Eng J Med *261* 882 889, 1959

35 *Morley, D* Severe measles in the tropics—I Brit med J *I* 297-300 1969

36 *Naconeczna I & Kay, S* Fatal disseminated cytomegalic inclusion disease in an adult presenting with a lesion of the gastrointestinal tract Amer J clin Path *47* 124-128 1967

37 *Nanada C N & Praparaj K C* Mediastinal emphysema. Indian J Ped at *36* 80-81 1969

38 *Pinkerton H Smiley W L & Anderson W A D* Giant cell pneumonia with inclusions. A lesion common to Hecht s disease distemper and measles. Amer J Path *21* 1 15 1945

39 *Quinn J L* Measles pneumonia in an adult. Amer J Roentgenol *91* 560 563, 1964

40 *Roberts G B S & Bain A D* The pathology of measles J Path Bact *I* 111-118 1958

41 *Shedden W I H & Emery J L* Immunofluorescent evidence of respiratory syncytical virus infection in cases of giant cell bronchiolitis in children. J Path Bact *89* 343 347, 1965

42 *Sincovics J G Trujillo J M Pienta R J & Ahearn M J* Leukemogenesis stemming from autoimmune disease International conference on leukemia lymphoma Ann. Arbor

Michigan Oct 1967 Ed Chris J D Zara fonetis, Philadelphia 1968 p 138–190

43 *Smythe, P M, Schonland, M, Brereton-Stiles, G G, Coovadia, H M, Grase, H J, Loening, B E K, Mafojane, A, Parent, M A & Vos, G H* Thymolymphatic deficiency and depression of cell mediated immunity in protein calorie malnutrition Lancet *II* 939 944, 1971

44 *Thunold S & Moe, P J* Complications of cytostatic therapy in childhood leukemia Acta path microbiol Scand Sect A Suppl *236*, 84–96, 1973

45 *Tietze, K W & Friedersdorff, F K* Riesenzellpneumonie nach Masern im Kindesalter beim mit Endoxan behandelten Reticulumzell Sarkom Z Kinderheilk *90* 341–250, 1964

46 *Warthin, A S* Occurrence of numerous large giant cells in the tonsils and pharyngeal mucosa in the prodromal stage of measles Arch Path *11* 864–874, 1931

47 *Wilson, C S* Measles as an universal disease Amer J Dis Child. *103* 219–223, 1963

48 *Woodruff, M* Immunsuppression and its complications Proc Roy Soc Med *62* 411–416, 1969

Acta path. microbiol scand Section A 81, 770–774, 1973

EXPERIMENTAL AMYLOIDOSIS

Studies of the Influence of Syngenic Parabiosis and Attempts at Transferring Experimental Amyloidosis via Parabiosis

F. HARDT and M H CLAESSON

University Institute of Pathological Anatomy and
University Institute of Medical Anatomy A, Copenhagen, Denmark

The development and transfer of casein induced amyloidosis was studied in parabiotic C_3H mice Induction of heavy amyloidosis in one of the members prior to the parabiotic connection did not result in transfer of amyloidosis to the healthy member even after treatment of the amyloidotic partner with nitrogen mustard—an agent known to accelerate the development of amyloidosis The development of casein induced amyloidosis following 20 injections of casein could be effectively inhibited by the performance of parabiosis with a healthy mouse in the middle of the casein treatment i e the pyroninophilic phase (*Teilum* 1964) The results are discussed in the light of recent hypotheses concerning the immunological nature of amyloidosis

Many clinical and experimental data suggest a close relationship between disturbances in the immune function and the occurrence of amyloidosis (conf *Teilum* 1964) Studies of casein-induced amyloidosis in mice and guinea pigs strongly suggest that alterations in the cellular immune system are of pathogenetic importance to the development of amyloidosis while the humoral immune functions in the amyloidotic animals seem intact (*Ranlov* & *Jensen* 1966, *Hardt* & *Claësson* 1971, *Hardt* & *Claësson* 1972a, *Cathcart et al* 1971)

In the present experiments we have studied the casein induced murine amyloidosis in parabiotic mice, as this model offers great advantages to the study of some crucial problems concerning the immunological nature of the disease Thus, the aim of the study has been to clarify whether cells capable of transferring the disease from amyloidotic to healthy mice are non-circulating lymphoid cells only located in the amyloidotic spleen as suggested from experiments by *Werdelin* & *Ranlov* 1966 and *Hardt* 1971, or whether these cells also circulate in the peripheral blood Another aim has been to examine the influence of an intact immune apparatus to the development of amyloidosis

Received 11 iv 73 Accepted 4 vi 73

Requests for reprints should be addressed to Dr *Finn Hardt*, University Institute of Pathological Anatomy, Juliane Maries vej 16 Copenhagen 2100 Ø, Denmark

MATERIAL AND METHODS

The animals were randomly selected inbred C_3H mice of both sexes aged 2 to 3 months at the beginning of the experiment

Parabiosis The parabiosis operation was performed between mice of equal sex and age A peritoneal flank incision was made on each animal and peritoneum was sutured to peritoneum and skin to skin Six days after the operation the effectivity of the parabiosis was checked by injecting one of the partners with a 0 2 ml of a 0 1 per cent solution of nigrosin If parabiosis was established

TABLE 1 *Experimental Design*

Group	Days of casein treatment* (A mouse)		Number of days† in parabiosis	Number of parabionts
	Prior to parabiosis	*After parabiosis*		
A	0	0	25	5
B	0	20	25	10
C	10	10	25	7
D	30	0	30	4
	30	0	15§	4

* Single mice injected with 0 5 ml 5 per cent sodium caseinate, parabiotic A mice injected with 1 0 ml 5 per cent sodium caseinate

† The day of operation = day 1 (see text)

§ A mouse treated with nitrogen mustard 5,3, and 1 days prior to sacrifice

the un injected partner would be coloured within 1 minute Only parabiosis with such established parabios s were used in the following experiments

Injection procedures All injections were given to the right parabiont which in the following will be referred to as the A mouse In principle daily subcutaneous injections of a 0 5 ml of a 5 per cent solution of sodium caseinate were given to single mice, while the A mouse in parabiosis received 1 0 ml per injection, i e the parabionts received the same amount of casein per Gm body weight as single mice did In order to accelerate the development of casein induced amyloidosis we used injections of 0 5 mg nitrogen mustard (Erasol®) 5, 3 and 1 day prior to sacrifice as proposed by Teilum (1954)

Experimental design The experimental design appears from Table 1 Group A consisted of un treated mice kept together in parabiosis for 25 days Group B consisted of parabionts where the A members received 20 injections of casein Group C consisted of parabionts where the A mice prior to parab osis recieved 10 casein injections and after parabiosis had been performed another 10 injections of casein Group D consisted of parabiotic mice where the A mouse prior to the operation had been treated with 30 injections of casein In this group some of the A members were treated with nitrogen mustard (see above)

Histological procedures At the end of the experiments the animals were killed with ether and the spleen, liver and kidney were fixed in neutral formaline Sections were stained with H E, methyl green-pyronine, alcaline Congo Red and PAS stain Amyloid was identified by its birefrigence with Congo Red under crossed polarizing filtres The degree of amyloidosis was evaluated on sections of spleens according to the semiquantitative method—ranging from 0 6—described by Christensen & Hjort (1959)

RESULTS

The histological events leading to deposits of amyloid in the spleen of mice receiving multiple injections of casein have been described in detail by Teilum (1956) and Christensen & Rask-Nielsen (1962) Briefly, after 10 injections of casein the spleens show intense perifollicular proliferation of pyroninophilic lymphoid cells concomitant with a marked decrease in the number of small lymphocytes After 20 injections of casein the pyroninophilia decreases and large numbers of PAS positive mesenchymal cells appear At this stage about 80 per cent of the spleens have deposits of amyloid substance located in a perifollicular position

As it appears from Table 2, parabiosis as such for up to 25 days does not induce pyroninophilic or amyloidotic changes in any of the parabiotic members (Group A) On the other hand, amyloidosis was found in spleen liver and kidney of both members in parabionts where the A mice had received 20 injections of casein (Group B) Table 2 furthermore shows that the development of casein induced amyloidosis can be effectivly suppressed in the A member if the parabiosis is performed in the middle of the casein treatment period i e after ten days' treatment (Group C) In this group of mice both parabionts remained in the pyroninophilic state with no signs of amyloid deposits The effec

TABLE 2 *Results*

Group	Amyloidosis							
	Spleen				Liver		Kidney	
	Frequency		Degree*		Degree†		Degree†	
	A mouse	B mouse	A mouse	B mouse	A mouse	B mouse	A mouse	B-mouse
A	0	0	0	0	0	0	0	0
B	8/10	7/10	2–3	2–3	sparse	sparse	sparse	sparse
C	0/7	0/7	0	0	0	0	0	0
D	4/4	0/4	3–4	0	heavy	0	heavy	0
	4/4§	0/4	4–5	0	heavy	0	heavy	0

* The method of Christensen & Hjort (1959) ranging from 0–6
† Three degrees 0, sparse, and heavy
§ A mouse treated with nitrogen mustard 5, 3, and 1 days prior to sacrifice

tiveness of circulating blood cells to transfer amyloidosis from heavily amyloidotic partners to untreated mice by parabiosis was studied in parabionts where the A mouse had received 30 injections of casein prior to the establishment of parabiosis (Group D) No signs of amyloidosis were ever seen in the B mouse, even if the A member had been treated with 3 injections of nitrogen mustard in order to accelerate the amyloid formation (*Teilum* 1954) In this group the A-member had spleen amyloidosis of degree six

DISCUSSION

I The development of amyloidosis in parabiotic animals is dependent on the antigenic incompatibility between the parabionts Thus genetically identical parabionts will not develop amyloidosis even after months of parabiosis (*Arras & Thierfelder* 1962, *Williams* 1964), whereas antigenic incompatibility between the parabionts will lead to amyloid formation in both partners, unless the antigenic incompatibility is so great that parabiotic intoxication will kill the animals within the first weeks of parabiosis Parallel to this we did not find any amyloidosis or deaths in our untreated homozygotic parabionts.

It has been shown by *Letterer* (1968) in guinea pigs that the casein induced amyloidosis in parabionts is strongly dependent on the dosis of casein per injection Thus, he could not induce amyloidosis in parabionts using the standard dosis for single animals However, an increase in casein dosis resulted in amyloidosis in both parabionts Taking advantage of this observation we induced amyloidosis using the same amount of casein per Gm body weight of single animals and parabiotic pairs, respectively

Inhibition of amyloid formation during casein treatment has been achieved by *Ranløv* (1967) with antilymphocyte-serum (ALS) simultaneously with casein treatment. *Ranløv* explained this as a result of a suppression of the cellular immune response, necessary for the initiation of the cellular events leading to amyloid formation This inhibitory effect of a depression of the cellular immune function is in contrast to the results of recent experiments according to which casein induced amyloidosis was accelerated in neonatally thymectomized mice and mice with congenital absence of the thymus (*Ebbesen* 1971, *Hardt & Claësson* 1972b) The result of the amyloid inhibition experiment by *Ranløv* might be explained as some effect of the ALS used on the mesenchymal cells which are known to produce amyloid (*Zucker Franklin & Franklin* 1970)

Hardt et al (1972) tried to inhibit the

amyloid formation by restoring the immune system They used weekly injections of healthy lymphoid cells into mice undergoing case in treatment Their efforts, however, failed and no inhibition was obtained—on the contrary, some acceleration of amyloid formation was found In the present experiment, inhibition of casein induced amyloidosis was obtained if parabiosis was established between the casein treated mouse (10 injections) and a healthy animal It has been shown that ten days of casein treatment decrease the ability of spleen cells to induce graft-versus host (GVH) reactions in F_1 hybrids (*Hardt* & *Claesson* 1971) and increase the number of decaying lymphoid cells in the spleen (*Claesson* & *Hardt 1972)* Furthermore recent evidence suggests that the thymus dependent cellular immune system is severely altered alredy after 10 injections of casein because at this time the number of cortisol resistent thymocytes—which are of major importance in GVH reactions (*Blomgren* & *Andersson* 1969)—is reduced to 25 per cent of the normal values (Claesson et al 1973) The establishment of parabiosis at this stage of casein treatment probably enforce the immune system and thereby postpone the break down of the immune apparatus which seems to be a mandatory step in the amyloid formation

II It has been shown by several workers that amyloidosis can be transferred by spleeen cells and fractions hereof to normal recipients (*Werdelin* & *Ranløv* 1966 *Janigan* & *Druet* 1968 *Hardt* 1971) However, the transfer of amyloidosis has been unsuccessfull by lymph node cells, thoracic duct lymphocytes, thymocytes, bone marrow cells, and blood lymphocytes (*Hardt* 1971) An explanation of these negative results might be that the kinetics of intra venously transferred lymphoid cell suspensions are quite abnormal compared to the normal kinetics of circulating lymphoid cells If there exists cells in the peripheral blood, capable of transferring amyloidosis, the most physiological way of tracing such cells seems to be in parabiotic animals In the present experiment, where heavily amyloidotic mice were connected with normal syngenic mice in parabiosis, amyloidosis was not identified in the B mouse, even after treatment with nitrogen mustard, known to accelerate the amyloidotic development (*Teilum* 1954) Thus, it might be concluded that the transferring agent in amyloidosis is restricted to non-circulating cells in the amyloidotic spleen The most likely candidate for transfer of amyloidosis seems to be an RE cell type for following two reasons First, ultrastructural studies have shown that RE cells can produce amyloid (*Zucker-Franklin* & *Franklin* 1970) Second, the number of RE cells in the spleen of amyloidotic animals is greatly increased (*Kazimierczak* 1969)

This work was supported by a grant from the *Danish League Against Rheumatism*

REFERENCES

Arras, H & *Thierfelder, S* Parabiose und Amyloidose Frankfurter Zeitschrift fur Pathologie 72 63–74, 1962

Blomgren, H & *Anderson, B* Evidence for a small pool of immunocompetent cells in the mouse thymus Exp Cell Res *57* 185–192, 1969

Cathcart, E S, *Mullarkey, M* & *Cohen, A S* Cellular immunity in casein induced amyloidosis Immunology *20* 1001–1008 1971

Christensen, H E & *Hjort, G H* X irradiation as accelerating factor in caseinate-induced amyloidosis in mice Acta path microbiol scand *47* 140–152, 1959

Christensen H E & *Rask Nielsen R* Comparative morphologic, histochemical and serologic studies of the pathogenesis of casein induced and reticulosarcoma induced amyloidosis in mice J Nat Cancer Inst *28* 1–33 1962

Claesson M H & *Hardt, F* Quantitative studies on the decay of lymphoid cells during the development of casein induced murine amyloidosis Acta path microbiol scand A *80* 125–133, 1972

Claesson M H, *Hardt F* & *Ropke, C* Amyloidosis and cellular immunity Local GVH reactions induced by combination of thymus and bone marrow cells or lymph node cells from mice challenged with various doses of casein 1973 to be published

Druet R L & *Janigan, D T* Experimental amyloidosis Amyloid induction with a soluble protein antigen in intact bursectomized and

thymectomized chickens. Amer J Path 49 1103-1123, 1966

Ebbesen, P Amyloid induction with casein in mice of different ages and investigation for casein antibodies using the single radial diffusion technique Virchows Arch Abt. B Zellpath 7 263-268, 1971

Hardt, F Transfer amyloidosis I Studies on the transfer of various lymphoid cells from amyloidotic mice to syngenic non amyloidotic recipients and II Induction of amyloidosis in mice with spleen, thymus and lymph node tissue from casein sensitized syngenic donors Amer J Path 65 411-422, 1971

Hardt, F & Claesson, M H Graft versus host reactions mediated by spleen cells from amyloidotic and non amyloidotic mice Transplantation 12 36-39, 1971

Hardt, F & Claesson, M H Quantitative studies on the T-cell population in spleens from amyloidotic and non-amyloidotic mice Immunology 22 677-683, 1972a

Hardt, F & Claesson, M H Studies on casein induced amyloidosis in mice with congenital aplasia of the thymus Acta path microbiol scand A 80 471-476 1972b

Hardt, F, Ebbesen, P & Moesner, J The effect of syngenic transfer of normal lymphoid cells on the development of casein induced amyloidosis in mice Acta path microbiol scand A 80 468-470 1972

Kazimierczak, K J Cytochemical study of casein induced and nitrogen mustard accelerated amyloidosis in mice Acta path microbiol scand 77 201-211 1969

Letterer, E Experimental amyloidosis with particular reference to parabiosis and to qualitative and quantitative changes of the amyloid inducing substances in mice and guinea pigs. *Amyloidosis* Proc Symp Amyloidosis. Excerpta Medica Foundation Amsterdam 1968 pp 361-367

Ranlov, P The role of the thymus in experimental mouse amyloidosis Acta path microbiol scand. 67 42 54, 1966

Ranlov P Effects of heterospecific antiserum against lymph node cells on the development of experimental amyloidosis in mice Acta path. microbiol scand 69 375-383, 1967

Ranlov, P & Jensen, E Homograft reaction in amyloidotic mice Acta path microbiol scand. 67 161-164, 1966

Teilum, G Studies on pathogenesis of amyloidosis J Lab clin Med 43 367-374, 1954

Teilum, G Periodic acid Schiff positive reticuloendothelial cells producing glycoprotein Functional significance during formation of amyloid. Amer J Path 32 945-959, 1956

Teilum, G Pathogenesis of amyloidosis The two-phase cellular theory of local secretion. Acta path microbiol scand 61 21-45, 1964

Werdelin, O & Ranlov, P Amyloidosis produced in mice by transplantation of spleen cells from casein treated mice Acta path microbiol. scand 68 1-18 1966

Williams, G Amyloidosis in parabiotic mice. J Path Bact 88 35-41, 1964

Zucker Franklin, D & Franklin E C Intracellular localization of human amyloid by fluorescence and electron microscopy Amer J Path. 59 23-41, 1970

NECROTIZING ANGIITIS PRODUCED BY THE SHWARTZMAN MECHANISM

KNUT NORDSTOGA and MORTEN FJOLSTAD

The National Veterinary Institute, Oslo, and the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo, Norway

Renal haemorrhagic and necrotic lesions, ranging from widespread haemorrhages to fulminant cortical necroses, were produced in experimental animals from 5 out of 9 mammalian species which received systemic injections of disintegrated cells of haemolytic *Escherichia coli* In animals exhibiting obvious macroscopic kidney lesions, necrotic mural alterations were consistently found in interlobular arteries and often also in afferent glomerular arterioles The acute vascular damage was frequently characterized by fibrinoid necrosis of the vessel walls, and was inconstantly accompanied by thrombosis and intramural and perivascular accumulations of inflammatory cells, predominantly eosinophils and mononuclear cells The vascular injury was interpreted as a type of hypersensitivity angiitis, and indicates that hypersensitive mechanisms are operative in the generalized Shwartzman reaction and in the development of bilateral renal cortical necrosis.

Thrombotic occlusions of interlobular arteries of the kidneys, afferent arterioles and glomerular capillaries are well established in association with the generalized Shwartzman reaction (GSR) This severe coagulopathy is by many authors regarded as the essential factor in the development of the bilateral cortical necrosis (BCN) which frequently accompanies the GSR (4, 9, 11, 21, 27) Less attention has been paid to the lesions in the arterial walls The purpose of this paper is to describe the mural changes of the renal arteries, associated with the GSR The reaction was elicited by systemic administration of disintegrated cells of *Escherichia coli* a material which in previous experiments had proved to be a potent Shwartzman mediator in pigs (26) The report is based on studies of animals from 9 different species of mammals Acute necrotic arterial lesions were found in one or more visceral organs in animals from all species, although such alterations were not present in renal arteries in all the species As the kidneys are considered to be target organs in the GSR (27), only renal arterial modifications are reported in detail in this paper

Received 16 v 73 Accepted 9 vi 73

Requests for reprints should be addressed to *Knut Nordstoga*, National Veterinary Institute, PB 8156, Oslo-Dep, Oslo 1, Norway

MATERIALS AND METHODS

The strain of haemolytic *Escherichia coli* type 0 141 a b (NVH 2653) was isolated from pig intestine The bacterial suspension was prepared by the same procedure and diluted to the same concentration as described previously (26) Experimental animals were of both sexes and differing ages, the number of experimental animals of each species is shown in Table 1 Except for some animals which died after one injection all animals received two intravenous, or intracardial injections, of varying sizes spaced 24 hrs apart Surviving animals were killed by intravenous, or intraperitoneal, injections of mebumal 24 48 hrs after the last inoculation All animals were autopsied, pieces of tissues were fixed in a 10 per cent formaldehyde solution for

TABLE 1 *Animals Injected with Disintegrated Cells of* Escherichia coli

Species	Number of animals/cases with evident gross renal damage
Blue fox (*Alopex lagopus*)	4/4
Ferret (*Mustela putorius furo*)	6/2
Mink (*Mustela vison*)	18/6
Rabbit (*Oryctolagus cuniculus*)	8/5
Pig (*Sus scrofa*)	3/1
Guinea pig (*Cavia porcellus*)	10/0
Chinchilla (*Chinchilla laniger*)	5/0
Dog (*Canis familiaris*)	2/0
Mice (*Mus musculus*)	20/0

at least 3 days, embedded in paraffin and sectioned at about 5 μ Sections were stained with haematoxylin and eosin (H & E), elastin van Gieson (el v G), periodic acid Schiff, Lepehne's haemoglobin stain phosphotungstic acid haematoxylin (PTAH), Lendrum's acid picro-Mallory method selected sections also with the Martius scarlet blue (MSB) method (8) One representative animal of each species, displaying obvious macroscopic renal damage and mural arterial modifications in the kidneys was selected as illustrative material

RESULTS

Most animals developed severe dyspnoea within half an hour after the injections, some of them gradually going into shock after the last injection Vomiting was almost without exception observed in pigs, mink, ferrets and dogs

Macroscopic Renal Lesions

1 Blue foxes In this species, the renal changes consisted only of widespread cortical haemorrhages without distinct limitations

2 Ferrets and mink In ferrets and mink, the affected kidneys had multiple infarct-like lesions or distinct haemorrhagic foci, alternating with pale areas in the cortex

3 Rabbits and pig Affected rabbit kidneys had multiple infarct like cortical lesions or total BCN, BCN was also present in the pig which had considerably enlarged kidneys with completely necrotic and haemorrhagic cortical layer, the kidneys being surrounded by subcapsular accumulations of blood

4 Other species Obvious gross renal lesions were lacking in guinea pigs, chinchillas, dogs and mouse

Estimations of macroscopic renal involvement of the animals on which the illustrations are based are shown in Table 2

Microscopic Renal Arterial Lesions

Mural modifications, of varying degree, were found in all animals with obvious gross renal changes, whereas arterial lesions were lacking in all individuals which did not reveal macroscopic renal alterations The major renal vessels appeared unchanged in all animals

1 Blue foxes This species did not display severe renal arterial lesions, although careful search revealed early changes in interlobular arteries and afferent glomerular arterioles in all animals, consisting predominantly of medial degeneration and transmural penetration of red blood cells (Fig 1) The arteries were frequently dilated, thrombi were not recognized

2 Ferrets and mink Mural arterial lesions in interlobular arteries and afferent arterioles

Fig 1 Incipient mural degeneration and subendothelial accumulation of erythrocytes (arrow) Interlobular artery of blue fox H & E, × 450

Fig 2 Mural necrosis and occlusive thrombosis in an interlobular artery of ferret. H & E, × 570

Fig 3 Mural necrosis with slight intramural and perivascular infiltrations of inflammatory cells occlusive thrombosis. Interlobular artery of ferret Staining el v G × 350

Fig 4 Mural necrosis with incipient intramural and perivascular infiltrations of inflammatory cells fragmentation of internal elastic membrane together with occlusive thrombosis in a distended interlobular artery Ferret staining el v G × 450

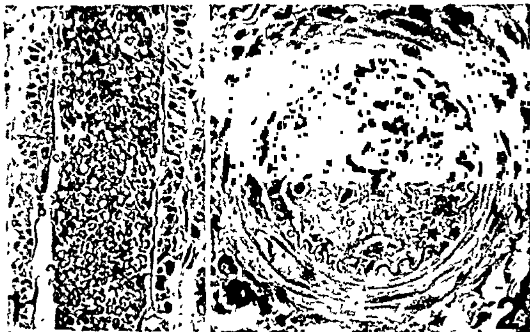

2
3
4

TABLE 2 *Treatment and Renal Changes in the Animals from which the Illustrative Material is Derived*

ecies	Route of challenge*	Age	Sex	Doses, ml First inject	Second inject	Killed (K) or died (D) hrs. after the second inject		Gross renal lesions
e fox	i v	Adult	♀	2 0	1 0	D	15	Scattered cortical haemorrhages
rret	i c	Adult	♂	1 0	1 0	K	48	Multiple infarct like lesions
ink	i c.	Adult	♀	0 5	0 3	K	24	Multiple infarct like lesions
bbit	i v	8 weeks	♀	0 4	0 4	K	48	BCN
g	i v	8 weeks	♀	3 5	4 0	K	48	BCN

*i.v = intravenous i c. = intracardial

were considerably more pronounced than in the foxes Incipient damage consisted of degeneration or necrosis of the smooth muscular elements of the media, with ensuing homogenization of the coat With increasing degree of involvement, the endothelial layer and the inner elastic membrane were more or less affected, the latter structure sometimes being discontinuous or fragmented (Figs 2 6) Scattered areas with fibrinoid* material were occasionally observed within the damaged vessel walls Massive transmural penetration of erythrocytes and intramural and/or periarterial accumulations of inflammatory cells occurred in both species, the inflammatory cells being predominantly eosinophils and mononuclear cells Thrombi were frequently found, the major component was, as a rule, PAS positive and stained partly as fibrin, but platelet and erythrocytic components could also sometimes be identified within the thrombo-occlusive material, and sometimes were also leucocytes entangled in the thrombi Minor fibrinous deposits were sporadically recognized in glomerular capillaries.

3 Rabbits and pig The most extensive arterial damage was found in the rabbit and the pig where the great majority of the interlobular arteries and the glomerular arterioles were affected The arterial lesions consisted of mural necrosis with extensive transmural penetration of red cells, occlusive thrombosis and intramural and/or perivascular infiltrations of eosinophils and mononuclear inflammatory cells Mural necrosis however, occurred frequently also in the absence of thrombosis (Figs 7 8) The necrotic vascular walls exhibited, as a rule, the characteristics of fibrinoid necrosis, and the major component of the thrombi had frequently the tinctorial properties of fibrin although red blood cells and leucocytes were also at times

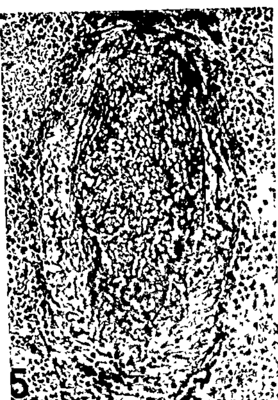
* The term "fibrinoid is used in this paper for the description of material within arterial walls which stained as fibrin with routine histochemical methods, without determination of origin

Fig 5 An interlobular artery of ferret Necrotic vascular wall and slight intramural and periarterial infiltrations of inflammatory cells (predominantly eosinophils) Occlusive thrombosis, the major part of the thrombus is stained as fibrin MSB × 260

Fig 6 Excentric lesion in an interlobular artery of mink mural necrosis with fragmentation of internal elastic membrane together with occlusive thrombosis. Staining el v G, × 350

Fig 7 Mural necrosis of an interlobular artery of rabbit Massive transmural penetration of red cells and occlusive thrombosis. H & E, × 570

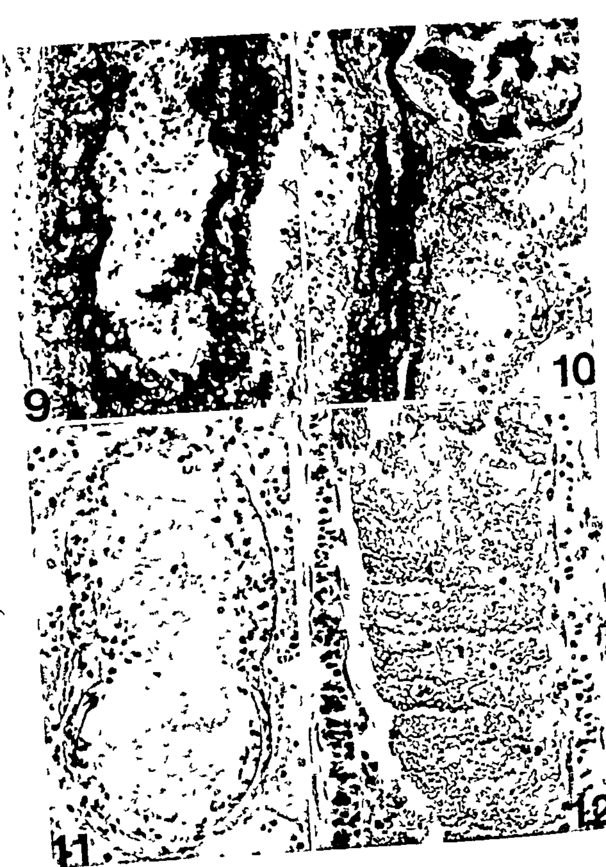
Fig 8 A necrotic interlobular artery of pig with massive intramural infiltration of eos nophils. Aggregates of leucocytes, entangled in a loose mesh of fibrin stained threads line parts of the necrotic vessel wall H & E, × 450


5

6

7

8


9
10
11
12

included, the thrombi adhered partly to the necrotic endothelium or fused with the fibrinoid material within the damaged arterial walls Massive fibrinous deposits were very common findings in glomerular capillaries (Figs 9 10) The internal elastic membrane was sometimes discontinuous, fragmented or totally destroyed, whereas the external elastica constantly was less damaged (Fig 11) Early mural alterations, without thrombosis, was only exceptionally observed (Fig 12)

4 Other species Any arterial modifications were not noted in guinea pigs, chinchillas, dogs and mouse

Extra renal Vascular Changes

Some individuals of each species which did not develop arterial lesions in the kidneys displayed necrotic lesions in extra renal arteries, predominantly in the lungs, sometimes with thrombosis However, venous thrombosis occurred also sometimes in these animals, pulmonary arterial lesions were sporadically observed also in animals with renal arterials lesions

DISCUSSION

The present experiment demonstrates that systemic administration of disintegrated cells of *Escherichia coli* induces acute necrotic arterial lesions in various species of mammals although species differences obviously exist in the morphological response to systemic applications of these materials In rabbits and the pig, the renal arterial changes were accompanied by extensive deposits of a fibrinous material within glomerular capillaries Fulminant BCN, the characteristic and identifying lesion of the GSR (*Thomas & Good* 1952) which classically is produced by two subsequent intravenous injections of bacterial endotoxins (4, 11, 27), was present in the same species

The acute necrotizing changes observed in arteries in various organs in the present investigation, correspond largely to the fibrinoid necrosis affecting medium-sized and small arteries in acute cases of polyarteritis nodosa, or to the group of acute necrotizing arterial lesions which *Zeek* and associates called hypersensitivity angiitis or allergic vasculitis (28-30) Such vascular injury is relatively common in various species in association with hypersensitivity to drugs and serum Arteries in the kidney and the lungs are frequently affected, sometimes are vessels in other viscera also involved (1, 15 18, 25, 28 30)

If induced by the Shwartzman mechanism, the BNC has mainly been explained on the basis of disseminated intravascular coagulation during recent years, this view receives support from many investigations in which factors influencing coagulation have been studied (3, 5, 10, 19, 20) However, vasomotor factors have also been pointed out as important in the pathogenesis of the GSR (2, 6, 11), and hypersensitivity has for many years by some authors been considered to be implicated in the development of experimental GSR, the action possibly depending on the occurrence of "natural' antibodies to endotoxins (23) Moreover, allergy to drugs has been noted in association with human cases of BCN (9, 22), the GSR has also been ob served after human transplantations (24) Experimentally, it has been shown that antigen-antibody complexes may precipitate the GSR in rabbits with 'blocked' reticulo endothelial system (7)

The present authors have previously induced GSR, including classical BCN and *renal arterial lesions equivalent to those re-*

Fig 9 Fibrinoid necrosis in an interlobular artery of pig PTAH × 450

Fig 10 A necrotic afferent arteriole and part of a glomerulus Vessel luminae are occluded by deposits, staining as fibrin. Pig, PTAH × 450

Fig 11 Necrotic interlobular artery with a partly destroyed internal elastic membrane and slight intramural infiltration of eosinophils Pig, staining el v G, × 450

Fig 12 Early lesion in an interlobular artery of pig Incipient intramural infiltrations of eosinophils at the left side. H & E, × 450

ported in this paper, in pigs (12, 13) and rabbits (14) by intravenous injections of crushed cells of *Haemophilus parainfluenzae* and *Salmonella cholerae suis*. Our observations demonstrate that suspensions of disintegrated cells of Gram negative bacteria are efficient Shwartzman mediators, our findings indicate also that hypersensitive reactions are involved in the pathogenesis of the GSR.

This work was supported by grants from the *Agricultural Research Council of Norway*

REFERENCES

1 *Citron, B P, Halpern, M, McCarron, M, Lundberg, G D, McCormick, R, Pincus, I J, Tatter, D & Haverback, B J* Necrotizing angiitis associated with drug abuse New Eng J Med. *283* 1003–1011, 1970

2 *Collins, A D, Henson, E C, Laird, S R & Brunson, J G* Norepinephrine, endotoxin shock, and the generalized Shwartzman reaction Arch Path *93* 82–88, 1972

3 *Good, R A & Thomas, L* Studies on the generalized Shwartzman reaction IV Prevention of the local and generalized reactions with heparin J exp Med *97* 871–888 1953

4 *Hjort, P F & Rapaport, S I* The Shwartzman reaction pathogenetic mechanisms and clinical manifestations Ann Rev Med *16* 135 168, 1965

5 *Kliman, A & McKay D G* The prevention of the generalized Shwartzman reaction by fibrinolytic activity Arch Path *66* 715 719, 1958

6. *Lambert, P F, Ming S C & Palmerio C* Vasomotor factors in the Shwartzman phenomenon Amer J Path *57* 559 580 1969

7 *Lee, L.* Antigen antibody reaction in the pathogenesis of bilateral renal cortical necrosis. J exp Med *117* 365–376 1963

8 *Lendrum, A C Fraser D C, Slidders, W & Henderson R* Studies on the character and staining of fibrin J clin Path *15* 401–419 1962

9 *Marcussen, H & Asnæs, S* Renal cortical necrosis. An evaluation of the possible relation to the Shwartzman reaction Acta path microbiol scand Section A. *80* 351–356 1972

10 *Margaretten, W & McKay D G* The role of the platelets in the generalized Shwartzman reaction. J exp Med *129* 585 590, 1969

11 *McKay, D G* Disseminated Intravascular Coagulation An intermediary mechanism of disease Hoeber Medical Division New York, 1965

12 *Nordstoga, K & Fjolstad, M* The generalized Shwartzman reaction and *Haemophilus* infections in pigs. Path vet *4* 245–253, 1967

13 *Nordstoga, K & Fjolstad, M* Porcine salmonellosis II Production of the generalized Shwartzman reaction by intravenous injections of disintegrated cells of *Salmonella cholerae suis* Acta vet scand *11* 370–379 1970

14 *Nordstoga, K & Fjolstad, M* Unpublished observations

15 *Nordstoga, K, Helgebostad, A, Loftsgård G & Stormorken H* Vesical hemorrhages in male Aleutian mink caused by hypersensitivity to sulphadimidine Acta vet scand *11* 481–483, 1970

16 *Rich, A R* The role of hypersensitivity in periarteritis nodosa as indicated by seven cases developing during serum sickness and sulphonamide therapy Bull Johns Hopk Hosp *71* 123 140, 1942

17 *Rich, A R & Gregory, J E* The experimental demonstration that periarteritis nodosa is a manifestation of hypersensitivity Bull Johns Hopk Hosp *72* 65–88, 1943

18 *Rich, A R* Studies on hypersensitivity Canad med Ass J *78* 163–169 1958

19 *Rodriguez Erdmann F* Production of the Sanarelli Shwartzman phenomenon by platelet factor 3 In Disseminated Intravascular Coagulation F K Schattauer Verlag, Stuttgart New York 1969 pp 63–65

20 *Shapiro, S S & MacKay, D G* The prevention of the generalized Shwartzman reaction with sodium warfarin J exp Med *107* 377–381, 1958

21 *Skjorten F* Bilateral renal cortical necrosis and the generalized Shwartzman reaction I Review of literature and report of seven cases Acta path microbiol scand *61* 394–404 1964

22 *Skjorten F* Generalized Shwartzman reaction Histopathological findings in six fatal cases with widespread lesions. Acta path microbiol scand *68* 517 534 1966

23 *Stetson C A* Role of hypersensitivity in reactions to endotoxin In Bacterial Endotoxins Proceedings of a symposium, Rutgers University, Quinn & Boden Co, Rahway, New Jersey 1964, pp 658–662

24 *Starzl T E Lerner R A Dixon, F J, Groth C G Brettschneider L & Terasaki, P I* Shwartzman reaction after human renal homotransplantation New Eng J Med *278* 642–648 1968

25 *Symmers W St C* The occurrence of angiitis and of other generalized diseases of con

nective tissues as a concequence of the administration of drugs (with a note on allergy as a cause of thrombotic purpura) Proc. royal Soc Med 20–28, 1962
26 *Teige, J jr, Nordstoga, K, Fjolstad, M & Nafstad, I* The generalized Shwartzman *reaction in pigs induced by diet and single* injection of disintegrated cells or partially puri fied endotoxin from *Escherichia coli* Acta vet scand *14* 92–106, 1973
27 *Thomas, L & Good, R A* Studies on the generalized Shwartzman reaction I General observations concerning this phenomenon J exp Med *96* 605 624, 1952
28 *Zeek, P M, Smith, C C & Weeter, J C* Studies on periarteritis nodosa III The differentiation between the vascular lesions of periarteritis nodosa and of hypersensitivity Amer J Path *24* 889-917, 1948
29 *Zeek, P M* Periarteritis nodosa A critical *review Amer J clin Path 22* 777 790, 1952
30 *Zeek, P M* Periarteritis nodosa and other forms of necrotizing angiitis New Eng J Med *248* 764–773, 1953

Acta path. microbiol scand Section A 81, 784–790, 1973

ANATOMICAL VARIATIONS OF THE CORONARY ARTERIES AND ORIGIN OF BLOOD SUPPLY TO SINOAURICULAR AND ATRIOVENTRICULAR NODES DETERMINED ON THE BASIS OF POSTMORTEM CORONARY ANGIOGRAPHY

STEIN PAULSEN and M VETNER

Patologisk Institut, Centralsygehuset, 7500 Holstebro, Denmark
(Chefer M Egholm & M Vetner)

On the basis of postmortem coronary angiography and macroscopic examination, the anatomical variations of the coronary arteries and origin of the blood supply to the sinus node and atrioventricular node was studied Four hundred coronary angiograms were performed of which 315 could be employed Anatomic variations of the coronary arteries were classified according to the system mentioned in Schlesinger's 1940 study In the present study we found 70 8 per cent of the coronary systems were of the right type 19 7 per cent of the balanced type and 9 5 per cent of the left type *Origin of the blood supply to* the sinus node (SN) was the right coronary artery in 63 8 per cent and the left coronary artery in 36 2 per cent Corresponding figures for the origin of the blood supply to the atrioventricular node (AVN) was 90 5 per cent from the right coronary artery and 9 5 per cent from the left coronary artery There was no statistically significant difference in *sex distribution*

Anatomical variations of the coronary arteries and variations in the blood supply to the sinus node (SN) and atrioventricular node (AVN) are important factors in the understanding of arrhythmias appearing in association with acute myocardial infarction (*James* 1968) As there are only a few studies on anatomical variations of the coronary arteries, we present here our findings in this regard which were obtained during a larger postmortem study of the heart

Definitions

We used *Schlesingers (1940)* classification of anatomical variations of the coronary arteries

Right type' refers to a coronary system where the right coronary artery supplies all of the free wall of the right ventricle and the posterior third of the interventricular septum and a greater or lesser part of the posterior wall of the left ventricle The remaining part of the myocardium of the left ventricle is supplied by the left coronary artery

Balanced type refers to a coronary pattern where the right coronary artery supplies the free wall of the right ventricle and the posterior third of the interventricular septum The rest of the myocardium of the left ven-

Received 18 ii 73 Accepted 16 vi 73

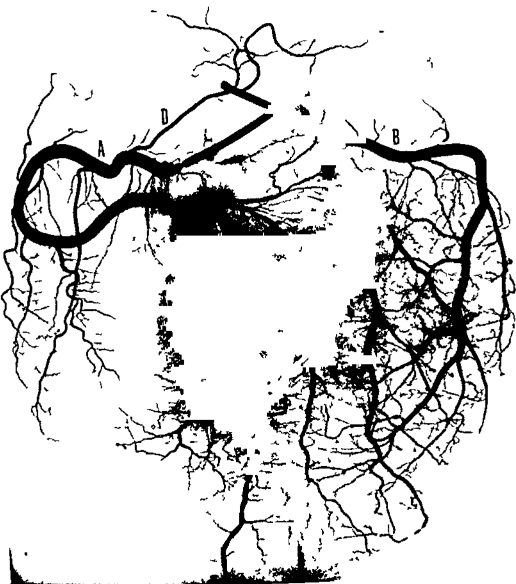


Fig 1 Right type A = right coronary artery B – left circumflex artery C – anterior descending artery D = s nus node artery E – pos erior descending artery

tricle is supplied by the left coronary artery

Left type refers to a coronary system where the left coronary artery supplies the free wall of the left ventricle and all of the interventricular septum together with a greater or lesser part of the posterior wall of the right ventricle The rest of the myocardium of the right ventricle is supplied by the right coronary artery

The arteriographic definition of these types is based on the degree of development of the right coronary artery and the circumflex branch of the left coronary artery With the right type the right coronary artery gives



Fig 2 Balanced type A = right coronary artery B = left circumflex artery C = anterior descending artery. D = sinus node artery E = posterior descending artery

off the posterior interventricular branch and branches to the posterior wall of left ventricle, whereas the left circumflex branch sends delicate branches to the lateral portion of the posterior wall of the left ventricle or in some cases the artery only reaches the obtuse margin (Fig 1).

In the "balanced type" the right coronary

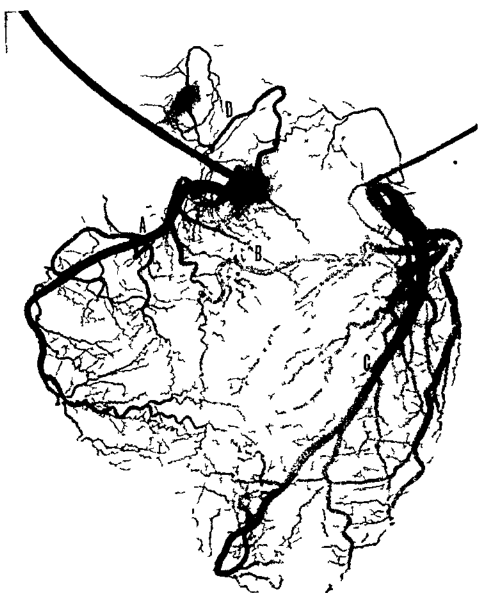


Fig 3 Left type A = right coronary artery B = left circumflex artery C = anterior descending artery D = sinus node artery

artery gives off the posterior interventricular branch whereas the left circumflex branch with its peripheral ramifications reaches the entire posterior wall of the left ventricle and gives off a large branch towards the apex parallel with the posterior interventricular branch (Fig 2)

The left type is a mirror image of the right type since the left circumflex branch gives off the posterior interventricular branch and can send branches (twigs) to the posterior wall of the right ventricle (Fig 3)

The arterial branch which supplies the SN arises either from the right coronary artery

or from the left circumflex branch. In most cases it arises from the first 1–2 cm of these two arteries, but in rare cases can arise from more peripheral portions. The blood supply to the AVN is dependent upon which artery supplies the posterior third of the interventricular septum.

MATERIAL AND METHODS

The study material was obtained by consecutive post mortem examinations of hearts in Ringkobing County (approx. 250 000 inhabitants) during a nine month period. Four hundred post mortem coronary angiographies were performed, 350 hearts were injected using manual pressure, whereas 50 were perfused with a constant pressure of 100 mm Hg and a constant temperature of 37° C. Perfusion time varied from 10 to 15 minutes, dependent upon the size of the heart.

After cleaning the cardiac cavities of blood, a catheter filled with contrast material was introduced into the ostia of the coronary arteries. The catheters were fixed in position with a suture, the end of the catheters being 5–10 mm from the coronary ostia. Barium sulphate (Mixobar® Astra) diluted 2:1 with water was used as contrast material. The right coronary artery was injected with blue material and the left with white.

The heart was photographed in the antero-posterior projection and from the side, the left ventricle being closest to the X-ray tube. The object was placed 80 cm from the tube and an exposure time of two seconds at 48–58 KV, according to the size of the heart, was used. RP/SX Omat RP 52 18 × 24 cm film (Kodak) was used and the X-ray machine was a Pleromobil 300 DP (Elma Schonander AB).

The only arteriograms used were those in which the entire coronary system was filled. With this criterion we were able to obtain 315 arteriograms. The rejected arteriograms came from patients with severe diffuse atherosclerosis of the coronary arteries or with occlusions which made it impossible to film all of the arteries. There were 49 men and 36 women in this group.

The chi square test was used for statistical calculations.

RESULTS

Table 1 tabulates per cent distribution of the anatomical pattern of the coronary arteries. In the study material there were 70.8 per cent "right type", 19.7 per cent "balanced type", and 9.5 per cent "left type". The difference between the distribution of men and women in the various groups was very slight.

TABLE 1 *Distribution of Coronary Artery Pattern*

	Total		Male		Female	
	No	Per cent	No	Per cent	No	Per cent
Right	223	70.8 (64–75)	133	71.1 (64–77)	90	70.3 (61–77)
Balanced	62	19.7 (15–25)	39	20.7 (15–27)	23	18.0 (11–25)
Left	30	9.5 (6–13)	15	8.0 (4–12)	15	11.7 (6–18)
Total	315	100.0	187	59.4	128	40.6

Bracketed figures indicate exact 95 per cent confidence limits

TABLE 2 *Origin of Sinus Node Blood Supply*

	Total		Male		Female	
	No	Per cent	No	Per cent	No	Per cent
Right	201	63.8 (57–68)	120	64.2 (56–71)	81	63.3 (54–71)
Left	114	26.2 (31–42)	67	35.8 (29–43)	47	36.7 (28–45)
Total	315	100.0	187	59.4	128	40.6

Bracketed figures indicate exact 95 per cent confidence limits

TABLE 3 *Origin of Atrioventricular Node Blood Supply*

	Total		Male		Female	
	No	Per cent	No	Per cent	No	Per cent
Right	285	90 5 (86–93)	172	92 0 (87–95)	113	88 3 (81–93)
Left	30	9 5 (6–13)	15	8 0 (4–12)	15	11 7 (6–18)
Total	315	100 0	187	59 4	128	40 6

Bracketed figures indicate "exact" 95 per cent confidence limits

TABLE 4 *Distribution of Coronary Artery Pattern (A) and Origin of Sinus Node Blood Supply (B)*

A	B	Total		Male		Female	
		No	Per cent	No	Per cent	No	Per cent
Right	(r)	147	46 7 (40–52)	88	47 0 (39–54)	59	46 1 (37–55)
Right	(l)	76	24 1 (18–28)	45	24 0 (18–31)	38	24 2 (16–32)
Left	(r)	20	6 4 (3–9)	11	5 9 (2–10)	9	7 0 (3–12)
Left	(l)	10	3 2 (1 6)	4	2 1 (1–5)	6	4 7 (1–9)
Balanced	(r)	34	10 8 (7–14)	21	11 2 (7–16)	13	10 2 (5–16)
Balanced	(l)	28	8 9 (6–12)	18	9 6 (5–14)	10	7 8 (3–13)
Total		315	100 0	187	59 4	128	40 6

Bracketed figures indicate "exact" 95 per cent confidence limits

and greatest for the "left type" where there were more women than men, but the difference between men and women in all groups was insignificant ($X^2 = 1\ 60$, 2 degrees of freedom, $P > 0\ 4$)

The blood supply of the SN is given in Table 2 Origin of the blood supply was the right coronary artery in 63 8 per cent and the left in 36 2 per cent The difference between men and women was very small and clearly insignificant ($X^2 = 0\ 055$, 1 degree of freedom, $P > 0\ 8$)

In Table 3 the blood supply to the AVN is given In 90 5 per cent of the cases it was supplied by the right coronary artery and in the remaining cases the blood supply came from the left coronary artery Blood supply from the right coronary artery was more common in men, whereas in women it was the left coronary artery, but the difference was not significant ($X^2 = 1\ 38$, 1 degree of freedom, $P > 0\ 2$)

In Table 4 variations in the coronary system are given together with origin of the blood supply to the SN As could be expected the most common combination was a "right type" with blood supply to the SN from the right coronary artery Differences between the various types in men and women were slight and none of them were of any statistical significance ($X^2 = 2\ 175$, 5 degrees of freedom, $P > 0\ 8$)

DISCUSSION

The present study was compared with three previous investigations (*Schlesinger* 1940, *James* 1961 and *Romhilt et al* 1968) *Schlesinger*s (1940) study delt with only anatomical variations in a consecutive, unselected autopsy material of 225 hearts It is not clear whether the results obtained in the study were based on arteriography alone or on a combination of macroscopic study and arteriography *James* (1961) carried out very careful anatomical studies of the coronary arteries using colored synthetic contrast materials and corrosion preparations The study

material was relatively small, 90 hearts with a somewhat unequal sex distribution From this study we have only been able to use results regarding blood supply to the SN and AVN *Romhilt et al* (1968) studied 192 hearts obtained from a selective autopsy material A combination of arteriography and macroscopic examination was used in this study and most of the patients had died of a serious heart condition

In comparison with our results *Schlesinger* (1940) found more hearts of the 'balanced type" and 'left type", 34 per cent and 18 per cent, respectively Sex difference was greatest in the group "left type" but calculations based upon the results presented show that sex difference was not statistically significant *Romhilt et al* (1968) found a greater number of hearts of the "right type" (81 per cent) whereas the 'balanced type" was only found in 3 2 per cent The difference between men and women was the same for both the "right type' and 'left type", there being 12 per cent more women with the "right type" and 12 per cent more men with the 'left type' Calculations based on the figures given in the tables show, however, the difference not to be statistically significant

As far as blood supplies to the SN there was a close correlation between our results and those of *Romhilt's et al* (1968) The latter found that blood supply to the SN in 60 8 per cent of the cases arose from the right coronary artery and 27 6 per cent of the cases from the left coronary artery where as in 1 6 per cent of cases it arose from both arteries In the study of *James'* (1961) there was a minor difference between the origin of the blood supply to the SN In 58 per cent of cases it arose from the right coronary artery and 46 per cent of cases from the left artery Sex differences given in these two studies were not significant and this is in agreement with our findings In both of the above mentioned studies hearts were found where the blood supply to the SN arose from both the right and the left coronary artery In our study we attempted to estimate which of these arteries was the largest and considered that blood supply arose from that artery The number of these cases is, however, so small that they have no statistical influence upon the results

Blood supply to the AVN was found by *Romhilt et al.* (1968) to arise from the right coronary artery in 84 7 per cent of cases and from the left coronary artery in 15 3 per cent of cases, whereas James' figures were 89 per cent and 11 per cent, respectively Calculations based on the results of the latter study demonstrate no statistical difference in sex distribution in accordance with our findings, whereas in the previous study there were more women in the group where the blood supply rose from the right coronary artery and this difference is statistically significant at the 5 per cent level, but not at the 1 per cent level

In the above mentioned studies comparison was not made between the anatomical variations of the coronary arteries and the origin of the blood supply to the sinus node as given in Table 4 This table shows that the most common combination was a "right type' with blood supply to the SN from the right coronary artery, whereas the rarest combination was the 'left type' with blood supply to the SN arising from the left coronary artery The difference between the distribution of men and women in these various types was not statistically significant in our study

---

This study was carried out with help from the *Medical Research Funds of the County Hospitals in Ringkobing Ribe and Sonderjylland*

REFERENCES

James T N Anatomy of the coronary arteries. Paul B Hoeber, New York, 1961

James, T N The coronary circulation and conduction system in acute myocardial infarction Prog Cardiovasc Dis *10* 410–449 1968

Romhilt D W, Hackel, D B & Estes Jr, E H Origin of blood supply to sinoauricular and atrioventricular node Amer Heart J *75* 279–280, 1968

Schlesinger M J Relation of anatomic pattern to pathologic conditions of the coronary arteries Arch Path *30* 403–415, 1940

Acta path. microbiol scand Section A 81, 791-805, 1973

DETERMINANTS FOR THE ESTABLISHMENT OF PERMANENT TISSUE CULTURE LINES FROM HUMAN GLIOMAS

B WESTERMARK, J PONTEN and R HUGOSSON

The Wallenberg Laboratory and The Department of Neurosurgery,
University of Uppsala, Uppsala, Sweden

Malignant gliomas, in contrast to other human neoplasms, rather frequently establish themselves as permanent tissue culture lines Still, the major part of malignant gliomas fail to do so The object of the present investigation was to search for determinants of the primary explant for the future successful establishment The material consisted of one medulloblastoma, one ependymoma, 4 oligodendrogliomas, 14 astrocytomas grade I and II and 68 astrocytomas grade III and IV Established lines were obtained only from grade III and IV gliomas but with no difference between these two groups A surprising finding was that male tumours more frequently became permanent lines (13/45) than female ones (1/23) and that a higher success rate was found among temporo parietal tumours (12/28 of the male tumours) than among those located in the frontal lobe (1/17 of the male tumours) No connection between the histologically predominant cell type of the grade III and IV gliomas and the rate of establishment was found Studies of the primary cultures of the gliomas implied that failure to form an established line only in a minor fraction could be ascribed to failure of the tumour cells of the explant to adhere to the solid support. This was valid for all astrocytomas grade I and II, the ependymoma and some of the grade III and IV gliomas In the majority of malignant gliomas a rapidly occurring failure to multiply was found, leading to continuous death of the tumour cells while normal glia like cells proliferated luxuriously and outnumbered the neoplastic component This might depend on a deficient interaction between the tumour cell and the solid support even if lack of essential metabolites cannot be excluded

Comparative growth control studies of normal and neoplastic human cells have for a long time been hampered by the unavailability of a suitable system Human neoplasms fail, with occasional exceptions, to form established cell lines* *in vitro* (*Moore* & *Koike* 1964) This has made it impossible to examine a large series of neoplasms of homogenous histogenetic origin

Our laboratory has developed a system which eliminates some of the above difficulties In apparent contrast to other human tumours, a high proportion of malignant brain tumours was found to give rise to established lines of tumour cell origin (*Ponten* & *Macintyre* 1968, *G Beckman et al* 1971) Even in this system, however, a majority of the explants failed to become established This report examines whether successful establishment is governed by chance fac-

* According to the nomenclature adopted by the American Tissue Culture Association the term "established cell line" is used for a cell line with infinite life span. By "cell line" is meant any serially cultivated culture

Received 12 ii.73 Accepted 23 vi.73

Requests for reprints should be addressed to *Bengt Westermark*, The Wallenberg Laboratory Box 562, 751 22 Uppsala 1, Sweden.

tors or can be linked to specific properties of the explants

MATERIAL AND METHODS

Initiation of Cultures

An unselected series of 88 neuro ectodermal tumours has been analysed Immediately after removal, the tumour specimen was placed in culture medium and transferred to the laboratory Grossly viable non haemorrhagic tumour tissue was freed from meninges and large vessels and minced with scissors The tumour material was vigorously pipetted and an appropriate amount of the suspension was seeded into 50 mm Falcon® Petri dishes and incubated

Control cultures were initiated in a similar way from brain tissue collected from operations on aneurysms, traumatic injuries etc

Maintenance of Cultures

During the first week the cultures were treated individually and the medium was changed when the pH of the medium had dropped After the initial phase, medium was renewed less frequently, i e twice a week The time both for the first and the subsequent subcultivations was chosen individually depending on the viability of the cultures, growth rate and proportion normal/tumour cells in order to favour neoplastic rather than non neoplastic cells

Eagle s minimum essential medium supplemented with 10 per cent baby calf serum was used after addition of 100 U penicillin/ml, 50 μg streptomycin/ml and 1 25 μg amphotericin B/ml The cultures were incubated at 37° C in humidified air, containing 5 per cent CO_2

For subcultivation (normally 1 2) medium was discarded and the cells were overlaid with a thin film of 0 25 per cent trypsin (Difco) and incubated at 37° C until they detached

Microscopic Examination of Cell Cultures

Living cultures were examined in the inverted microscope at least three times a week For staining, cultures were washed in phosphate buffered saline, fixed for 10 min in methanol and stained for 10 20 min in Giemsa's dye diluted 1 10 in distilled water

Histological Examination

After fixation overnight in formalin, embedding, sectioning and staining according to v Gieson, the tumours were classified using *Kernohan's et al* classification (1949) which distinguishes between astrocytoma grade I, II, III and IV

In addition to the standard classification, a "cytological" examination was performed on the sections The shape of the predominant cell type (see Figs 8-11) To reduce subjective errors, photomicrographs were taken from different representative fields of all tumours at the same magnification Homogenous areas without signs of necrosis oedema or inflammation were selected for the photographic recording The photographs were then treated as unknowns and distributed into different categories according to Table 3 The classification pattern could be reproduced in repeat tests with coded photocopies

RESULTS

Cultures of Non-neoplastic Brain Tissue

Successful cultures were started from 73/73 adult brain explants The main characteristics of normal glia-like cultures have been described (*Pontén & Macintyre* 1968, *Ponten et al* 1969) The primary yield of viable attaching elements was low from non neoplastic brain but 7-14 days after explantation scattered colonies of vividly migrating and proliferating cells were detected Most colonies were composed of polygonal or star-shaped, well-spread cells resembling simplified astrocytes A few colonies per dish consisted of elongated, fibroblast-like cells When, after 14-21 days, some 50 per cent of the bottom surface was covered by cells, subcultivation was started During the period of rapid growth (phase II, *Hayflick* 1965) the cultures could be transferred once or twice a week In all but one case, detectable fibroblasts disappeared during serial cultivation The exceptional line (363 CG) was entirely composed of fibroblast-like cells until it reached the end of its finite life span

All non-neoplastic cell lines had a finite life span ending in phase III degeneration (*Hayflick* 1965) after 20-30 passages

Cultures of Neoplastic Brain Tissue

General description Influence of sex, age and tumour localization on frequency of establishment of tumour lines The series of 88 tumours comprised one medulloblastoma, one

TABLE 1 *Fraction of Established Cell Lines in Relation to Sex of Patient, Localization and Histological Grade of the Gliomas*

Sex	Male			Female		
Localization of tumour	III	IV	Total	III	IV	Total
Frontal lobe	1/6	0/11	1/17	0/4	0/8	0/12
Temporal lobe	5/9	5/14	10/23	0/4	0/2	0/6
Parietal lobe	1/2	1/3	2/5	0/1	0/3	0/4
Occipital lobe	0/0	0/0	0/0	0/0	1/1	1/1
Total			13/45			1/23

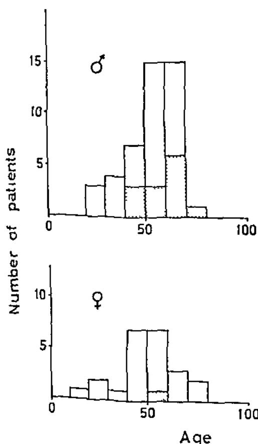


Fig 1 Age distribution of male (upper part) and female (lower part) patients bearing astrocytomas grade III or IV Empty bars indicate non established lines and dotted bars established lines Note similarity in distribution between the established and the non established groups

ependymoma, 4 oligodendrogliomas, one astrocytoma grade I, 13 grade II, 26 grade III and 42 grade IV Permanent cell lines were obtained only from grade III and IV astrocytomas

The frequency of establishment as a function of the donor's sex, localization of the tumour and the histological classification of the 68 grade III and IV astrocytomas appear in Table 1 One conspicuous finding is that the success rate among the male tumours was much higher (13/45) than that of the female neoplasms (1/23) From the figures of the male group it is evident that it was much easier to establish lines from tumours of the temporal (10/23 established) and parietal lobes (2/5 established) than from tumours of the frontal lobe (1/17 established)

The failure of the female tumours could, if at all, only partially be explained by a higher frequency of frontal lobe tumours in this group The only established female tumour was localized to the occipital lobe

The material of low degree astrocytomas was too small to allow any definite conclusion Since only 5 tumours belonged to the group of high potential frequency of establishment (i e male temporo-parietal tumours), the present results do not exclude the possibility that it could be possible in a larger material to establish lines from such tumours

The age distribution of patients whose tumours gave rise to established cell lines coincided grossly with that of the non established group (Fig 1)

No significant difference in success rate was found between grade III and IV astrocytomas

TABLE 2 *Properties of Prin*

Cell line designation	Sex	Diagnosis	Localization of tumour
278 MG	F	astro IV	T
281 MG	F	astro IV	P
286 MG	F	astro IV	F
289 MG	F	astro II	F
291 MG	M	astro IV	T
302 MG	F	astro III	P
313 MG	M	astro II	T
314 MG	M	astro IV	F
321 MG	M	astro III	T
324 MG	M	astro IV	F
327 MG	M	astro IV	F
329 MG	M	astro IV	P
341 MG	M	astro IV	T
343 MG	M	astro III	T
346 MG	M	astro III	P
348 MG	M	astro IV	T
362 MG	F	astro III	T
364 MG	M	astro IV	F
372 MG	M	astro IV	T
373 MG	M	astro IV	T
375 MG	M	astro IV	F
380 MG	F	astro IV	F
384 MG	M	astro II	F
385 MG	F	astro IV	F
388 MG	F	astro II	P
389 MG	M	astro II	F
395 MG	M	astro III	T
399 MG	M	astro III	T
401 MG	M	astro III	F
402 MG	M	astro III	F
407 MG	M	astro II	T
410 MG	M	astro III	T
414 MG	F	astro IV	T
430 MG	F	oligo	F
473 MG	M	oligodendro-glioblastoma	F
475 MG	F	astro I	T
482 MG	F	astro III	F
489 MG	M	astro IV	T
491 MG	M	astro II	T
495 MG	M	astro III	T
496 MG	M	astro III	P
497 MG	F	ependymoma	F
502 MG	M	astro IV	P

Abbreviations astro = astrocytoma. oligo = oligodendroglioma F = Frontal lobe P = Parietal lobe T = Temporal lobe

ltures of Human Gliomas

Primary* adhesion	Primary* migration	Primary* proliferation	Predominant cell type of primary culture	Established cell line
+ +	+ +	+	astrocytic	no
+ +	+ +	+	astrocytic	no
+ +	+ +	+	astrocytic	no
0				no
+	+	0	astrocytic	no
+ + +	+	+ +	small spindle cells	no
0				no
0				no
+	+	0	astrocytic	no
+	+	0	astrocytic	no
0				no
+ +	+	+	polygonal cells	no
+ + +	+ + +	+ +	astrocytic	no
+ +	+ + +	+ +	spindle cells	yes
+ + +	+ + +	+ +	astrocytic	no
+	+ +	+ +	spindle cells	yes
0				no
+ +	+ +	+	astrocytic	no
+ +	+ +	+ +	spindle and astrocytic	yes
+ + +	+ + +	+ + +	spindle	yes
0				no
+ +	+ +	+	astrocytic	no
0				no
+ +	+ +	+	astrocytic	no
0				no
0				no
+ + +	+ +	+	astrocytic	no
+ +	+ +	+	spindle cells	yes
0				no
+ +	+ +	+	astrocytic	no
0				no
+	+ +	+	spindle cells	yes
+ +	+ +	+	astrocytic	no
0				no
+ + +	+ + +	+ +	blastoid oligodendroglia like cells	no
0				no
0				no
+ +	+ +	+ +	spindle cells	yes
0				no
+ +	+ +	+	astrocytic + spindle cells	yes
0				no
0				no
+ +	+ +	+	astrocytic	no

* Only cells morphologically classified as "glioma cells" were considered *In all negative cases, primar* outgrowth of normal looking elements took place

Properties of the Primary Cultures

Lines 278 MG–502 MG were subjected to a particularly careful morphological and behavioural study from the time of explantation to the deterioration or establishment of the respective cell line (Table 2) The primary outgrowth (Table 2) of explanted tissue entailed three events 1 Attachment of tissue fragments and single cells to the solid support 2 Migration of cells from the attached fragments 3 Cell proliferation These three parameters were estimated under the microscope using both living and stained cultures

Attachment The degree and speed of primary attachment varied within wide limits between the different lines Grade I and II astrocytomas did not produce any attached cells of undisputable neoplastic origin On the other hand, in explants of grade III and IV gliomas at least a few tumour cells were almost always capable of adhering to the solid support

In a few cases, especially lines 343 MG and 346 MG, attachment was very rapid and after only a few hours a nearly confluent cell layer had formed Ordinarily, however, attachment was slow and did not end until a few days after explantation This was shown whenever free-floating tissue fragments form 3-4 days old cultures continued to attach after transfer to new dishes

One tumour (473 MG) consisted of rather monomorphic immature cells with a clear and light cytoplasm Judging from the morphology, the cells were of oligodendroglial origin The tumour was therefore classified as an oligodendroglioblastoma The donor was a fourteen-year old boy The tumour tissue was easily fragmented and the cells attached readily, yielding a confluent cell layer after a few days

The ependymoma (497 MG) was also easily dissociated A suspension of single cells and small multicellular aggregates was obtained but the cells were completely unable to attach to the solid support

Trypsinization increased the yield of attaching cells only in those lines which had cells capable of spontaneous attachment

Migration In most cases (Table 2) the rate of migration correlated to the amount of attached cells Generally, migration started very early, often within a few hours and always within the first two days after the cultures had been initiated

Proliferation It was difficult to discriminate between *de novo* attachment and proliferation during the first few days If, however, no free floating elements remained after a few medium changes, all increase in cell number could be ascribed to proliferation The presence of mitotic figures was also used as a sign of multiplication Except for three negative lines, glioma cell proliferation was detected in all lines with primarily attached tumour cells (Table 2)

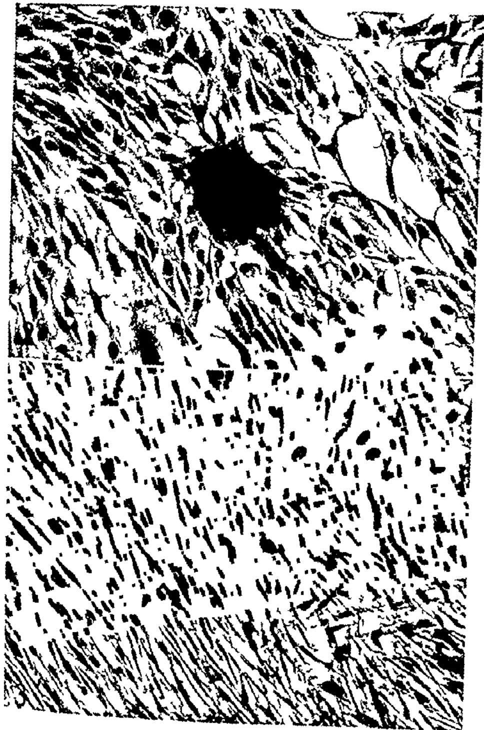
On the basis of the yield and fate of identifiable glioma cells the serially cultivated lines were divided into three groups

1 Lines with no identifiable tumour cells 2 Lines with primary outgrowth of glioma cells undergoing degeneration after some time *in vitro* 3 Lines with primary outgrowth of tumour cells giving rise to permanent cell lines composed of the neoplastic elements

Group 1 During the first days, only vacuolized macrophages and fragments of tumour tissue adhered to the culture vessel After one or two weeks, cells began to migrate from the attached fragments and mitoses

Fig 2 The centre of the figure shows an explanted piece of a glioma (496 MG) from which cells are migrating The thin cells are uniform in size with abundant cytoplasm and nuclei of a regular ovoid shape Their morphology is basically astrocytic. A few mitoses are seen in the monolayer The cells are indistinguishable from those derived from non neoplastic brain tissue (cf *Ponten and Macintyre* 1968 and *Ponten et al* 1969) Giemsa × 160

they were regarded normal fibroblasts (derived from perivascular tissue?) Giemsa × 160




4
5

were frequent These cells were identified as astrocyte like elements undistinguishable from glia like cells derived from non neoplastic brain (Fig 2) A few colonies per dish consisted of fibroblastic cells

During their whole life span, the cultures were composed of monomorphic astrocyte-like cells, except for two lines (289 MG and 314 MG) which were composed of elongated, fibroblastic cells (Fig 3), resembling 363 CG (*vide supra*) These cells were lost after 30-40 passages while the other cultures of group 1 were lost after 20 30 passages

Group 2 During the first week, the cultures were predominantly composed of cells of undisputable neoplastic origin The glioma cells were pleomorphic with irregular nuclei with unevenly dispersed chromatin The cytoplasm was often drawn out into long projections so that the cells formed a fibrillar network (Fig 4) Among these cells an increasing amount of multinucleated cells was detected (Fig 5) In a few cases, primary outgrowth of spindle or polygonal cells of neoplastic appearance took place

Mitotic figures were present only during the first few days After some 10 days no proliferation of tumour cells could be detected Simultaneously with the decrease in growth rate, the formation of projections was enhanced and the fibrillar structure of the culture was pronounced After the first few days, astrocyte like cells with a "normal' morphology started to migrate from the adhered tumour fragments Among these cells proliferation was lively and soon the glioma cells were outnumbered by normal glia-like cells Concomitantly with the multiplication of normal elements the tumour cells began to degenerate Pyknosis was often seen and fragmentation of whole cells took place After subcultivation, a large proportion of the tumour cells failed to resettle, leading to their rapid disappearance If the cultures were not transferred, tumour cells could persist for 1-2 months Attempts to enhance tumour cell survival by the use of a "complete" medium (Ham's nutrient medium F-10, *Ham* 1963) or foetal calf serum were in vain

302 MG behaved somewhat differently from the rest of the lines The primary cultures were composed of heaps of small, elongated cells with small oval hyperchromatic nuclei which did not migrate from the colonies Mitoses could sometimes be seen and slowly the volume of the heaps increased, still without any centrifugal migration Whenever such a culture was subcultivated, only a small proportion of the cells resettled and the tumour cells were rapidly lost If not subcultivated, the viability was retained and the tumour was maintained in primary culture for two years when it eventually became contaminated with mould

The growth pattern of the primary cultures of the oligodendroglioblastoma (473 MG) appears in Fig 6 Mitoses were seen only during the first few weeks Further proliferation could not be initiated by subcultivation After two months, degenerative changes started until complete cell death after eight months in culture

Group 3 The degree of primary attachment of the gliomas belonging to this group did not differ from that of the preceding group In contrast to group 2, however, all lines had a large proportion of fusiform cells with no or few, short ramifications (Fig 7) One line (373 MG) proliferated luxuriously from the very beginning and no normal glia cells were ever found The tumour cells of the other cultures started to multiply very slowly allowing normal elements to outnumber the glioma cells after some fourteen

Fig 4 Primary culture of 346 MG 9 days after explantation The explant is situated to the lower right (not shown in the Figure) The tumour cells have an astrocytic morphology with long cytoplasmic projections After some time these glioma cells degenerated, though looking very viable at the time of photographic recording, as described in the text Living culture × 160

Fig 5 Primary culture of line 395 MG grown for 14 days *in vitro* The culture is composed of atypical astrocytoid cells producing a rich network of cytoplasmic projections. Note nuclear polymorphism and multinuclear cells. Giemsa × 160

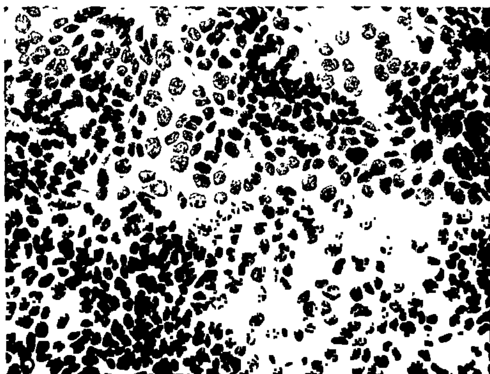
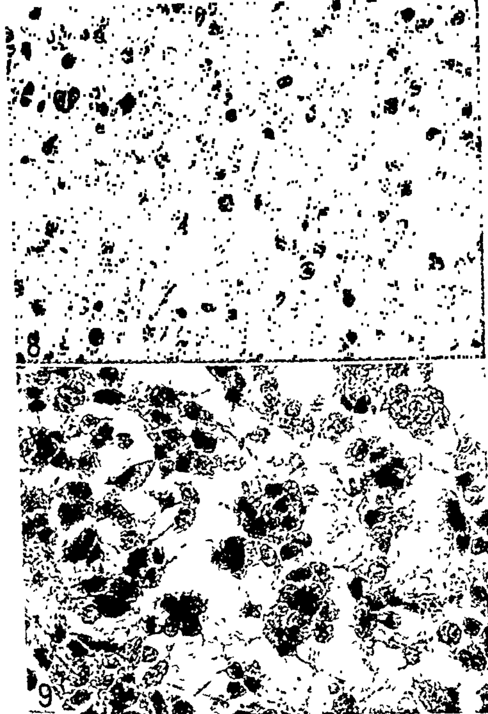




Fig 6 Primary culture of the oligodendroglioblastoma (473 MG) The microscopic field is filled with moderately pleomorphic, rather small cells arranged in heaps with suggestive alignment of nuclei in parallel rows In spite of the unusually high viability of the primary outgrowth this culture eventually succumbed to irreversible degeneration Giemsa × 160

Fig 7 Primary culture of 343 MG three weeks after explantation Spindle or polygonal tumour cells migrate out from the explant (lower right) Living culture × 160



Figs 8-11 Histological sections representing four different groups employed for the "cytological" classification of the astrocytomas grade III and IV (see also Table 3) Van Gieson × 650

Fig 8 shows a glioma composed of cells with a basic astrocytoid shape with an abundant fibrillar cytoplasm (AR according to Table 3)

Fig 9 In this glioma the cells also have an astrocyte like appearance but with a scanty cytoplasm (AP according to Table 3)

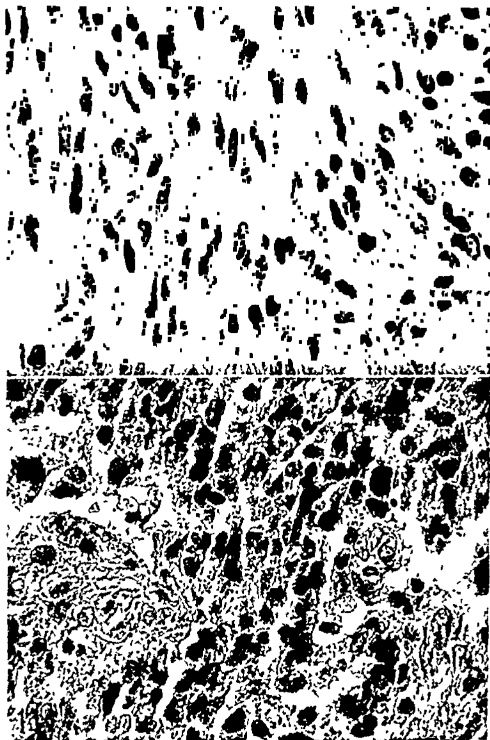


Fig 10 The section shows a glioma with fusiform cells with a moderate amount of fibrillar cytoplasm (BM according to Table 3)

Fig 11 Rather immature looking cells with a basic polygonal or round shape designated as *PP* in Table 3

TABLE 3 *Cytologic Classification of 68 Grade III IV Malignant Astrocytomas* Underscored *Numbers Became Established Tumour Cell Lines*

Basic astrocytoid shape Amount of cytoplasm			Basic bipolar shape Amount of cytoplasm			Basic polygonal shape Amount of cytoplasm	
rich AR	medium AM	poor AP	rich BR	medium BM	poor BP	rich PR	poor PP
67 MG	104 MG	83 MG	175 MG	73 MG	160 MG	70 MG	150 MG
87 MG	119 MG	*105 MG*	*251 MG*	88 MG	170 MG		166 MG
231 MG	168 MG	*138 MG*	258 MG	108 MG	286 MG		263 MG
239 MG	247 MG	147 MG		*118 MG*	364 MG		327 MG
401 MG	265 MG	161 MG	482 MG	120 MG	385 MG	0/5	
496 MG	346 MG	174 MG		127 MG	414 MG		
	362 MG	*178 MG*		135 MG			
	375 MG	203 MG		136 MG		One line 324 MG not possible to classify	
	380 MG	269 MG		144 MG			
	399 MG	281 MG		278 MG			
	402 MG	302 MG		291 MG			
	410 MG	314 MG		329 MG			
		321 MG		*343 MG*			
		341 MG		*348 MG*			
	8/35	*373 MG*		502 MG			
		395 MG		6/27			
		495 MG		(incl *372 MG* & *489 MG*)			

days Since normal glia cells are sensitive to contact inhibition of the cell cycle (*Ponten et al* 1969) their proliferation stopped when a confluent cell sheet was formed The capacity of the cultured glioma cells to multiply in the presence of stationary normal cells (*Westermark* 1974) rather rapidly altered the glia glioma cell relation and after a few subcultivations, glia cells were no longer de tected

Relation between Explant Cytology and Formation of Permanent Lines

(Representative pictures of the cytological groups appear in Figs 8 11)

Table 3 summarizes the distribution of the predominant cell type in the 68 grade III-IV malignant gliomas All but three lines (324 MG, 372 MG and 489 MG) were sufficiently uniform to permit unequivocal sub classification 324 MG was extremely pleomorphic and all morphological categories were found in the sections 372 MG and 489 MG were of the bipolar type (B) but could not be further subdivided because cells from all subcategories were present in roughly equal amounts

It is seen that no significant difference has been established between predominance of a particular morphologic cell type and capacity to become permanently established

Since no permanent lines were obtained from the biopsies composed of polygonal cells, it is possible, however, that these cells in a larger material would be found unsuitable for growth as surface attached cells in culture This possibility is strengthened by the failure of all six explants of biopolar cells poor in cytoplasm (BP) to become established since these cells are morphologically closely related to the polygonal cells

DISCUSSION

Permanent establishment of surface attached cell lines *in vitro* requires the following conditions in the explant 1 Presence of viable cells 2 Capacity of cells to survive the trau-

matic experience of explantation 3 Ability of surviving cells to thrive on a solid support 4 Capability of endless multiplication *in vitro*

All our glioma lines have contained viable neoplastic cells as judged from the macroscopic and microscopic appearance of the specimen obtained at operation This is supported by the finding of intact tumour cells in grids examined histologically early after explantation (*Hugosson* & *Westermark* 1974) In the same investigation it was found that most explants, in spite of rather heavy initial necrosis contained viable and multiplying areas of glioma cells for several weeks

We conclude that the major obstacle for establishment is not absence of viable cells or failure to survive the shock of explantation

A large proportion of the tumours failed to grow as permanent lines despite primary outgrowth of tumour cells and initial proliferation of such elements The reason for this is obscure but at least three explanations are possible

1 The nutrient medium is insufficient

2 The tumour cells cannot attach properly to the solid surface Since they may be anchorage dependent this will lead to necrosis even in a nutritionally adequate milieu

3 An inherent limited growth potential as in non neoplastic cells The last explanation is not likely because of the very short life span of the tumour cells—the cultures were not at all transferable One would rather expect that the cultures could be grown for at least a few passages and then degenerate if alternative 3 were true

The first possibility is difficult to exclude However use of Ham s F 10 instead of Eagle s medium did not enhance tumour cells survival In a few cultures foetal rather than baby calf serum was used without any improvement Furthermore comparison between survival of cells in the intact explants and the culture fluid revealed that viable proliferating neoplastic cells could remain in the intact explant maintained in an organ type grid culture in spite of a failure to form a permanent line on solid plastic (*Hugosson* & *Westermark* 1974) Taken together, these facts suggest that alternative 2, i e an incompatibility between the cell surface and the plastic support may be the most important factor in preventing long term growth This may either manifest itself primarily in which case no attachment at all takes place or after subcultivation when the trypsin may permanently remove essential cell coat components.

The capacity to attach is presumably absent from the low grade astrocytomas since the outgrowth from such explants did not differ significantly from that of non neoplastic brain With the reservation that the morphological difference between tumour and normal brain seen *in vivo* is completely obliterated *in vitro*, we have to conclude that the cells which attach and temporarily multiply are derivatives of normal rather than neoplastic glia Even if one assumes that the normally looking glia cells are instead grade I or II astrocytoma cells, there still remains a deficiency with respect to an infinite multiplication potential since no permanent lines were obtained

Grade III and IV astrocytomas could sharply be divided into two classes with regard to their capacity to form permanent lines

Unexpectedly this was strongly correlated with the sex of the donor and the location of the tumour As a reflection of this, 12 out of 28 male grade III IV astrocytomas of the parieto temporal region (43 per cent) gave rise to permanent lines *in vitro* This tendency has remained over a period of more than five years and has resulted in the highest proportion of successfully established human lines of proven neoplastic origin from a defined tumour on record

The reason for the peculiar inclination of male parieto [illegible] grade astrocytomas to cont[illegible]
plastic and [illegible]
obscure It does not seem to relate to any special clinical features in this group of patients

A conspicuous finding was the difference

in morphology of the primary outgrowth between the established and the non established group Since 8/9 of the explants which contained a large fraction of spindle cells became established lines and no permanent line was obtained from the astrocytic group, the presence of a large proportion of spindle cells seems to be an early and reliable sign of further establishment of the culture Unexpectedly, this had no connection with the *in vivo* cytology as reported by *Kersting* (1961) The reason for this discordance is obscure

A finding by *Kersting* (1961), confirmed by us, is that glia derived cells differentiate and achieve an astrocytic morphology with many long cytoplasmic projections if cultivated in a poor medium, e g in medium without calf serum which is unable to support cell proliferation Therefore, a pronounced astrocytic morphology of glioma cells might be due to their non proliferative state This could explain the above relationship between the explant morphology and establishment An alternative explanation is that only more dedifferentiated glioma cells are capable of *in vitro* life

In conclusion, the establishment of permanent cell lines from human gliomas was not a random event It was strongly dependent on the presence of a certain class of cells capable of primary attachment and survival after repeated subcultivations by trypsin This class was only present in astrocytomas grade III-IV Its morphological appearance *in vivo* could not be ascertained, it may in fact be multimorphic Also *in vitro* established lines had a variable morphology, however a bipolar cell resembling a primitive neuroblast was the most prevalent type (*Ponten & Macintyre* 1968) We suggest that a compatibility between the cell coat and the solid plastic support is the most important prerequisite for successful establishment The same putative cell coat factor may be responsible for attachment and maintenance of a bipolar morphology

For completely obscure reasons the class of cells capable of establishment under our culture conditions was practically confined to the parieto temporal region of male tumour patients

---

This investigation was supported by grants from the *Swedish Cancer Society* (55 B72 07XB) The skilful technical help by *Margareta Lindstrom* and *Marianne Pettersson* is gratefully acknowledged

REFERENCES

1 *Beckman, G, Beckman, L Pontén, J & Westermark, B* G 6-PD and PGM phenotypes of 16 continuous human tumor cell lines Human Heredity *21* 238–241, 1971

2 *Ham, R G* An improved nutrient solution for diploid Chinese hamster and human cell lines Exp Cell Res 29 515 526, 1963

3 *Hayflick, L* The limited in vitro lifetime of human diploid cell strains. Exp Cell Res *37* 614–636, 1965

4 *Hugosson, R & Westermark, B* Survival of human normal and neoplastic glia in matrix culture To be published

5 *Kernohan, J W, Mabon R F, Svien, H J & Adson, A W* A simplified classification of the gliomas Proc Staff Meet, Mayo Clinic *24* 71–75, 1949

6 *Kersting, Gunter* Die Gewebszuchtung menschlicher Hirngeschwulste Monographien aus dem Gesamtbebiete der Neurologie und Psychiatrie Eds. M Muller, H Spatz P Vogel Springer Verlag Berlin Gottingen, Heidelberg, 1961

7 *Moore, G E & Koike, K* Growth of human tumor cells in vitro and in vivo Cancer *17* 11–20, 1964

8 *Ponten, J & Macintyre E H* Long term culture of normal and neoplastic human glia Acta path microbiol scandinav *74* 465–486, 1968

9 *Pontén, J Westermark B & Hugosson, R* Regulation of proliferation and movement of human glia like cells in culture Exptl Cell Res *58* 393–400, 1969

10 *Westermark, B* Growth regulatory interactions between stationary human glia like cells and neoplastic cells in culture II Neoplastic cells To be published

Acta path microbiol scand Section A 81, 806–812, 1973

EXPERIMENTAL CARDIAC HYPERTROPHY

An Autoradographical Study after in vivo Injections of ³H-5-Uridine

GUNNAR UNGE

The Departments of Pathology and Thoracic Medicine, Karolinska sjukhuset, Stockholm, Sweden

Various forms of experimental cardiac hypertrophy were investigated by light and electron microscope autoradiography and measurements of myocardial tissue radioactivity after *in vivo* injections of ³H 5 uridine in rats In the autoradiograms, the degree of labelling of striated muscle cells and capillary wall cells was estimated A significant increase in ³H 5 uridine incorporation was recorded in the striated muscle cells of the left ventricle wall of rats with aortic stenosis, hypertension of short duration and of swimming exercised rats. In the latter case, an increased labelling was recorded also in the myocardial capillary endothelium In the hypertrophying heart, the uridine was found in the striated muscle cell mitochondria in higher percentage than in the controls

Increase in total myocardial RNA content has been recorded repeatedly in various forms of experimental cardiac hypertrophy (*Sumner & McIntosh* 1963, *Gluck et al* 1964 and *Korecky & French* 1967) In cardiac hypertrophy secondary to aortic stenosis this increase in RNA content can be ascribed to an increase in RNA synthesis activity (*Fanburg & Posner* 1968 and *Koide & Rabinowitz* 1969) Some of this increased RNA synthesis probably takes place in the interstitial cells (*Morkin* 1971)

In recent experiments, features were observed which were considered to indicate a significantly greater neo-formation of myocardial blood capillaries in cardiac hypertrophy induced by swimming exercise than in cardiac hypertrophy secondary to arterial hypertension and aortic stenosis, in the two latter conditions, a neo formation of myocardial capillaries was, in fact, questionable (*Ljungqvist & Unge* 1972, *Ljungqvist & Unge* 1973, *Mandache et al* 1972 and *Mandache et al* 1973) In these experiments, alterations were also found in the striated muscle cells of hypertrophied hearts such as increased numbers of mitochondria (*Mandache et al* 1972) These findings initiated the present study in which an attempt was made to investigate the degree of RNA synthesis in the hearts of rats with various forms of cardiac hypertrophy by light microscope, autoradiography, and measurements of myocardial tissue radioactivity after ³H 5 uridine injections For further identification of the labelled cells, electron microscope autoradiography of the hearts was also performed

Received 16 v 73 Accepted 18 vi 73

Requests for reprints should be addressed to *Gunnar Unge*, M D Department of Pathology, Karolinska sjukhuset, S 104 01 Stockholm, Sweden.

MATERIAL AND METHODS

Female Sprague Dawley rats were used in the investigation They were fed a standard laboratory diet containing 0 4 per cent sodium chloride and

tap water *ad libitum* Cardiac hypertrophy was induced by the production of renal hypertension, aortic stenosis and by swimming exercise (*Ljungqvist* & *Unge* 1972). The animals were grouped as follows

Group I Eight rats in which renal hypertension was produced by the application of an 0 15 mm wide silver clip on the left renal artery The blood pressures were measured with the tail pletysmographic method at daily (Group I A) or weekly intervals (Group I B) Hypertension developed within 2–6 weeks The animals were divided into two subgroups

I A Four animals which were killed within the first week of hypertension and at a terminal weight of 230 ± 5 g (mean ± SD)

I B Four animals which were killed 3 months after the development of hypertension and at a terminal weight of 280 ± 5 g

Group II Twelve animals in which an 0 5 mm wide silver clip was applied to the immediate sub diaphragmatic part of the aorta They were divided into three subgroups

II A Four animals which were killed 5 days after the operation and at a terminal weight of 250 ± 5 g

II B Four animals which were killed 2 weeks after the operation and at a terminal weight of 250 ± 0 g

II C Four animals which were killed 2 months after the operation and at a terminal weight of 255 ± 5 g

Group III Twelve animals were subjected to swimming exercise for 1 hour each day, 6 days per week. They were divided into three subgroups

III A Four animals which were killed after the second week of exercise and at a terminal weight of 250 ± 5 g

III B Four animals which were killed after 4 weeks' exercise and at a terminal weight of 255 ± 5 g

III C Four animals which were killed after 4 weeks exercise followed by a resting period of 4 weeks and at a terminal weight of 255 ± 0 g

Group IV This group consisted of 4 normal rats the age of which was chosen to make them suitable as controls for the rats of group I A, the terminal weight was 230 ± 0 g

Group V This group consisted of 4 normal rats the age of which was chosen to make them suitable as controls for the rats of groups II A–C and III A–B They also served as controls for the slightly older rats of groups I B and III C the terminal weight was 250 ± 0 g

Three hours before death, 2 μ Ci/g body weight of ^{3}H 5 uridine (specific activity 26 4 Ci/mM, NEN) was injected intraperitoneally At the end of the experimental periods, the rats were anaesthetized by an intraperitoneal injection of Nembutal The aorta was exposed and a catheter inserted and directed towards the heart Fixation of the heart was then performed by perfusion *via* the catheter with 1 5 per cent glutaraldehyde solution buffered by Na cacodylate to pH 7 4 During this procedure the animals died The hearts were removed and weighed for determination of the heart/body weight ratios In Table 1 these ratios are multiplied by 1000 for practical reasons One half of each heart was taken for light microscope autoradiography and the other half, except in groups II A, III C and IV, for electron microscope autoradiography

Light Microscope Autoradiography

The material was post fixed in 4 per cent glutaraldehyde solution and, after paraffin embedding, 4 μ thick transverse sections were taken from the ventricle portions of the heart and placed on glass slides The sections were washed with 5 per cent ice cooled trichloro-acetic acid (TCA) and tap water and after drying covered with Kodak AR 10 film for autoradiography according to the stripping technique The films were exposed for 9 weeks at +4° C, developed in Kodak D 19 B and fixed in Kodak acid fixer The sections were then stained through the film with haematoxylin-eosin The autoradiograms were examined under the high power oil immersion lens in an ordinary binocular light microscope For evaluation of the degree of uridine incorporation into the tissue, the grain density of striated muscle cells and capillary wall cells was determined according to a scale ranging from + (normal) to + + + (heavily increased) The entire material was coded to make the examiner ignorant of the history of each case

Tissue Radioactivity

In order to obtain information about the uridine content in the hearts, 0 5 mm thick sections were taken from each paraffin block and three circular pieces with a diameter of 2 mm were pinched out from the left ventricle wall These pieces were incubated in 1 ml soluene (Packard) at +40° C for 18 hours after which 15 ml of toluol POP (0 5 per cent) + POPOP (0 05 per cent) was added and the counts per minute (c p m) were determined in a liquid scintillation spectrometer (Tri Carb model 3003 Packard)

Electron Microscope Autoradiography

After 24 hours in 4 per cent glutaraldehyde, pieces of the left ventricle wall were postfixed in 1 per cent OsO_4 buffered at pH 7 4 and embedded in Epon 812 The sections were not washed with TCA since this might damage the material The material was processed for electron microscope auto-

radiography according to the methods described by *Salpeter & Bachman* (1964) and *Bachman & Salpeter* (1967) with some modifications (*Mandache et al* 1973) Four ribbons of thin sections from each block were taken These were placed on droplets of uranyl acetate for 1 hour at +60° C and lead citrate for 5 minutes after which they were transferred to collodion coated glass slides (*Galey & Nilsson* 1966) The slides were carbonized and an Ilford L4 emulsion monolayer was applied by the dipping technique to the purple interference colour The material was exposed for 12–13 weeks at +4° C, followed by development in G 230 (Agfa Gevaert) for 90 seconds and transfer of the slides to a stop bath containing 1 per cent acetic acid The slides were then washed, fixed by G 305 (Agfa Gevaert) for two minutes and washed again in distilled water Five minutes later the collodion film was stripped off on a water surface while it was still not completely dry Copper grids were placed on the ribbons and picked up for drying The sections were examined in a Siemens Elmiskop I and the cell structures related to the grains were analysed



Fig 1 Light microscope autoradiogram of left ventricle wall of hypertrophied rat heart (swimming exercise 2 weeks) showing labelled capillary wall cell *(arrow)* and *grains* related to the striated muscle cell cytoplasm A number of capillary lumina (L) are seen and these are practically devoid of grains while grains are seen in a capillary wall cell (arrow) × 2080

RESULTS

Table 1 shows that a significant increase in heart weight occurred in *all* experimental groups In the group of rats in which exercise was followed by rest, the increase in heart weight was less pronounced than in the exercised rats that did not rest

TABLE 1 *Effect of Renal Hypertension, Aortic Stenosis and Swimming Exercise on the Heart Weight (Ratio) of Rats*

Group	Dur	Age	Weight	B p	Ratio
I A	1 wk	3 5	230±5	195±10	4 5±0 1*
I B	3 mos	6 5	280±5	180±15	5 7±0 7§
II A	5 days	4 5	250±5	115±10	4 6±0 2§
II B	2 wks	4 5	250±0	110±10	5 0±0 1§
II C	2 mos	4 5	255±5	110± 5	5 1±0 1§
III A	2 wks	4 5	250±5	115±10	5 6±0 3§
III B	1 mos	4 5	255±5	115±10	5 7±0 5§
III C	1 + 1 mos	5 5	255±0	120±15	4 8±0 2§
IV		3 5	230±0	110± 5	4 1±0 1
V		4 5	250±0	110±10	4 1±0 2

Group I = renal hypertension Group II = aortic stenosis Group III A–B = swimming exercise Group III C = swimming exercise followed by rest Group IV and V = control rats Dur = duration of experimental situation B p = blood pressure at end of experiment Ratio – heart weight × 1000/body weight The figures are means ± SD

* Significantly different from control groups (0 001 < p < 0 01)

§ *Significantly different from control groups* (p < 0 001)

TABLE 2 *Labelling Indices of Striated Muscle Cells, Capillary Wall Cells, Striated Muscle Cell Mitochondria and Tissue Radioactivity in Left Ventricle Wall after ³H 5 Uridine Injections in Rats with Various Forms of Cardiac Hypertrophy*

Group	Light microscope autoradiography		c p m	Mitochondria
	grains of striated muscle cells	grains of capillary wall cells		
I A	+ + +	+ +	140±13§	78 %
I B	+	+	87± 4§	26 %
II A	+ + +	+ +	156±12§	–
II B	+ + +	+ +	137± 4§	90 %
II C	+	+	89± 3§	73 %
III A	+ +	+ + +	111± 4§	59 %
III B	+ +	+ + +	100± 6§	43 %
III C	+	+	75± 4*	–
IV	+	+	70± 3	–
V	+	+	64± 6	14 %

Group I = renal hypertension Group II = aortic stenosis Group III A–B = swimming exercise Group III C = swimming exercise followed by rest Group IV and V = control rats. + = normal density + + = increased density + + + – heavily increased density c p m. = counts per minute The back ground activity measured on non injected material was 26±7 Mitochondria percentage of grains related to mitochondria – = not investigated The figure are means ± SD
* Significantly different from control groups (0 02 < p < 0 01)
§ Significantly different from control groups (p < 0 001)

The grain density determined on the light microscope autoradiographs is seen in Table 2 If compared with the corresponding control rats (normal density), a heavily elevated grain density was recorded in the striated muscle cells in the hearts of the short term hypertensive rats and rats with aortic stenosis of short duration (Fig 1) A less marked in crease in grain density was recorded in the striated muscle cells in the hearts of rats that did not rest after terminated exercise As can be seen in Table 2, the grain density of capil lary wall cells was increased in the exercised rats that did not rest and less so in the rats with aortic stenosis and hypertension of short duration

It can further be seen from Table 2 that the tissue radioactivity was increased in all experimental groups The most pronounced increase was observed in the group of rats sacrificed 5 days after constriction of the aorta In the other groups of rats with constricted aorta the tissue radioactivity de creased with the prolongation of the experimental period but was still significantly ele vated 2 months after the operation A significant increase in tissue radioactivity was also found in the animals with short term hyper tension, and this too became lower with prolongation of the experimental situation, although it was still significantly elevated after 3 months as compared to the controls In the swimming exercised animals, a more constant elevation of tissue radioactivity was found A slight increase was still present after one month's rest following the termination of exercise

In electron microscope autoradiograms, the grains were seen to be distributed mainly in the cytoplasm of the striated muscle cells In the control animals, most grains were scattered throughout the cytoplasm and 14 per cent of the grains was related to the mitochondria (Table 2) In the experimental animals, on the other hand, the proportions of grains related to mitochondria were higher (Fig 2) Thus, as much as 90 per cent of the grains was related to mitochondria in the rats with aortic stenosis sacrificed 2 weeks after the operation and 73 per cent in the rats sacri-

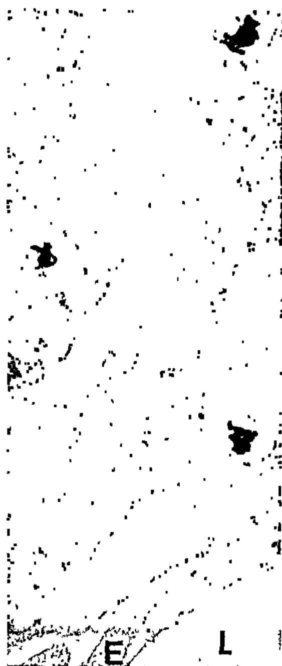


Fig 2 Electron microscope autoradiogram of heart section from rat with aortic stenosis (2 weeks) showing labelled striated muscle cell mitochondria Capillary lumen (L) Endothelial cell (E) × 26 500

ficed 2 months after the operation. The percentage of grains related to mitochondria was also high in the short term hypertensive animals (78 per cent), but this percentage markedly decreased after 3 months (26 per cent). In the swimming exercised rats a moderately increased percentage of grains related to mitochondria was found (59 per cent and 43 per cent).

Few grains were found in the endothelial cells and most of these grains were scattered throughout the cytoplasm A slight increase in the number of grains was found in the exercised rats that did not rest, but not in the other experimental groups.

DISCUSSION

Several biochemical processes are accompanying the development of cardiac hypertrophy The DNA synthesis is increased and this can be ascribed to a proliferation of the interstitial cells (*Morkin* & *Ashford* 1968 and *Grove et al* 1969) Recent investigations have shown that the capillary wall cells constitute the main proliferating tissue component, this proliferative activity is particularly pronounced in cardiac hypertrophy secondary to swimming exercise (*Ljungqvist* & *Unge* 1973 and *Mandache et al* 1973)

In cardiac hypertrophy there is a parallel increase in heart weight and protein content per heart (*Sumner* & *McIntosh* 1963, *Gluck et al* 1964 and *Korecky* & *French* 1967). Since RNA of various types participates in protein synthesis, the increase in the rate of RNA synthesis that has been shown to take place in cardiac hypertrophy secondary to aortic stenosis (*Fanburg* & *Posner* 1968 and *Koide* & *Rabinowitz* 1969) indicates an activated protein synthesis

In the present investigation, the content and distribution of the RNA-precursor was determined in the myocardial tissue both by measurements of tissue radioactivity and by light microscope autoradiography following in vivo injections of the labelled compound into rats with various forms of cardiac hypertrophy The cellular and subcellular distribution of the uridine was further analysed by electron microscope autoradiography By these methods an increased uridine content was demonstrated in the hypertrophied

hearts, and this was mainly located in the cytoplasm of the striated muscle cells. It is known that the degree of uridine labelling is not necessarily a measure of RNA synthesis In the present determinations of tissue radioactivity, acid soluble nucleotides were probably included since this material was not treated with cooled trichloro-acetic acid (TCA) In the light microscope autoradiograms of TCA treated sections, the pool of acid soluble nucleotides was probably less important and the labelling of the myocardial cells therefore more relevant It thus appears that an increased RNA and, consequently, protein synthesis probably occurred in the striated muscle cells of the hearts of the experimental animals, at least during the developmental phases of the hypertrophy In the abscence of an increased DNA synthesis (*Ljungqvist* & *Unge*, and *Mandache et al* 1972), the findings are in agreement with the general view that cardiac hypertrophy results from an increase in size and not in number of striated muscle cells (*Meesen* 1971)

In the rats with hypertension and aortic stenosis, the uridine labelling was more pronounced in the short term experimental animals (Groups I A, II A and II B) than in the long term groups (I B and II C), suggesting that the process of hypertrophy was more intense in the former three groups of animals than in the latter two This is supported by the fact that the degree of cardiac hypertrophy was significantly more pronounced after 3 months of hypertension than after 1 week ($0 01 < p < 0 05$) as it was after 2 months of aortic stenosis than after 5 days and 2 weeks ($0 001 < p < 0 01$ for both groups) In the long term groups, however, the hypertrophying process was still taking place to a certain degree, to judge from the increased tissue radioactivity in these groups of rats as compared with the controls.

In the swimming exercised rats, no differences in tissue radioactivity and autoradiographical pictures were recorded whether the rats had been exercised for 2 weeks or 1 month Nor was there any difference in heart weight ratios between these groups of rats Thus, the most intense phase of the hypertrophying process had obviously taken place before the end of the second week of exercise A certain degree of continuing hypertrophy was, however, still taking place both after 2 weeks and 1 month of exercise, to judge from the autoradiograms and tissue radioactivity of these hearts as compared with the control rats

The size and number of the mitochondria of the striated heart muscle cells have been found to increase during periods of prolonged increase in myocardial work load (*Wegner* & *Mölbert* 1966, *Meessen* 1971 and *Mandache et al* 1972) It has also been shown that mitochondria contain their own DNA and RNA (*Mazur* & *Harrow* 1971), and it is probable that cardiac enlargement involves stimulation of mitochondrial nucleic acid and protein synthesis (*Rabinowitz et al* 1971) Increase in the mitochondrial RNA has been found in cardiac hypertrophy secondary to aortic stenosis (*Ito* 1968), and increased incorporation of leucine into the striated muscle cell mitochondria has been demonstrated in hearts made hypertrophic by aortic stenosis and swimming exercise (*Breithard et al* 1969, *Hamberger et al* 1969 and *Shahab* & *Wollenberger* 1970) In the present investigation, the labelling of the striated muscle cell mitochondria was highly increased in the groups of animals in which the hypertrophying process was going on This further emphasizes the importance of the function of the striated muscle cell mitochondria in the hypertrophying heart

The ^{3}H 5 uridine labelling was also increased in the myocardial capillary wall cells of the swimming exercised rats that did not rest and to a less extent, in the aortic stenosis animals and short term hypertensive animals This is in good agreement with our previous observation of increased numbers of ribosomes in capillary endothelial cells of hypertrophied hearts (*Mandache et al* 1972) and is a further indication of the growth activity of the capillary endothelium in su (*Ljungqvist* & *Unge* 1972, *Mo* 1972 and *Mandache et al* 197

This study was supported by the *Swedish Medical Research Council* (Project No B72 12X 716 07A) the *Swedish National Association against Heart and Chest Diseases* and the *Research Funds of Karolinska Institutet*

REFERENCES

1. *Bachman, L* & *Salpeter, M M* Absolute sensitivity of electron microscope radioautography J Cell Biol *33* 299–305, 1967
2. *Breithard, G*, *Hilscher, W* & *Poche, R* Autohistoradiographische Untersuchungen uber den Einbau von H 3 Leucin in den hypertrophierten Herzmuskel nach Schwimmtraining Arch Kreislaufforschung *59* 351–369, 1969
3. *Fanburg, B L* & *Posner, B J* Ribonucleic acid synthesis in experimental cardiac hypertrophy in rats Circ Res, *23* 123–135, 1968
4. *Galey, F R* & *Nilsson, S E G* A new method for transferring sections from the liquid surface of the trough through staining solutions to the supporting film of a grid J Ultrastruct. Res *14* 405–410, 1966
5. *Gluck, L*, *Talner, N S*, *Stern, H*, *Gardner, T H* & *Kulovich, M V* Experimental cardiac hypertrophy Concentrations of RNA in the ventricles Science *144* 1244–1245, 1964
6. *Grove D*, *Zak, R*, *Nair, K G* & *Aschenbrenner, V* Biochemical correlates of cardiac hypertrophy IV Observations on the cellular organization of growth during myocardial hypertrophy in the rat Circ Res *25* 473–485, 1969
7. *Hamberger, A*, *Gregson, N* & *Lehninger, A L* The effect of acute exercise on amino acid incorporation into mitochondria of rabbit tissues Biochim Biophys Acta *186* 373–383 1969
8. *Ito, Y* Protein and nucleic acids of rabbit heart muscle in experimentally produced aortic stenosis Jap Circ J *33* 1805–1807, 1968
9. *Koide, T* & *Rabinowitz, M* Biochemical correlates of cardiac hypertrophy II Increased rate of RNA synthesis in experimental cardiac hypertrophy in the rat Circ Res *24* 9–18, 1969
10. *Korecky B* & *French, I W* Nucleic acid synthesis in enlarged hearts of rats with nutritional anemia Circ Res *21* 635–640, 1967
11. *Ljungqvist, A* & *Unge, G* The finer intramyocardial vasculature in various forms of experimental cardiac hypertrophy Acta path microbiol scand Sect A *80* 329–340, 1972
12. *Ljungqvist, A* & *Unge, G* The proliferative activity of the myocardial tissue in various forms of experimental cardiac hypertrophy Acta path microbiol scand Sect A *81* 233–240, 1973
13. *Mandache, E*, *Unge, G* & *Ljungqvist, A* Myocardial blood capillary reaction in various forms of cardiac hypertrophy An electron microscopical investigation in the rat Virchows Arch Abt B Zellpath *11* 97–110, 1972
14. *Mandache, E*, *Unge, G*, *Appelgren, L E* & *Ljungqvist, A* The proliferative activity of the heart tissues in various forms of experimental cardiac hypertrophy studied by electron microscope autoradiography Virchows Arch. Abt B Zellpath *12* 112–122, 1973
15. *Mazur, A* & *Harrow, B* Textbook of biochemistry W B Saunders Company (London), 1971, pp 399–402
16. *Meessen, H* Structural bases of myocardial hypertrophy British Heart J *33* 94–99, 1971
17. *Morkin, E* Myocardial nucleic acid metabolism in experimental cardiac hypertrophy Cardiac hypertrophy by Normal R Alpert, Academic Press (New York), p 259–271, 1971
18. *Morkin, E* & *Ashford, T P* Myocardial DNA synthesis in experimental cardiac hypertrophy Am J Physiol *215* 1409–1413, 1968
19. *Rabinowitz, M*, *Aschenbrenner, V*, *Albin, R*, *Gross, N J*, *Zak, R* & *Nair, K G* Synthesis and turnover of heart mitochondria in normal hypertrophied and hypoxic rat Cardiac hypertrophy, by Normal R. Alpert Academic Press (New York), p 283–299, 1971
20. *Salpeter, M M* & *Bachman, L* Autoradiography with the electronmicroscope A procedure for improving resolution, sensitivity and contrast J Cell Biol *22* 469–475 1964
21. *Shahab, L* & *Wollenberger, A* Amino acid incorporation into mitochondria from hypertrophying hearts of rats with aortic constriction J Molecul Cell Cardiol *1* 143–155 1970
22. *Sumner, R G* & *McIntosh, H D* Nucleic acid studies in experimental cardiomegaly Circ Res *12* 170–175, 1963
23. *Wegner, G* & *Molbert, E* Des Verhalten des Myokards bei der experimentellen supravalvularen Aortastenose Autoradiographische und elektronenmikroskopische Untersuchungen an Rattenherzen Virchows Arch path Anat. *341* 54–63 1966

Acta path microbiol scand Section A 81, 813-823, 1973

LUNG TUMOURS IN ICELAND

JONAS HALLGRIMSSON

The Department of Pathology, University of Iceland Reykjavik, Iceland
(Head Ólafur Bjarnason)

Two hundred and twenty three primary lung tumours submitted for histological diagnosis in Iceland during the period 1941–1968 were typed according to the WHO histological classification The distribution of the four major types was epidermoid carcinomas 20 6 per cent (males 26 4 per cent females 10 1 per cent), small cell anaplastic carcinomas 37 2 per cent (males 39 6 per cent, females 32 9 per cent), adenocarcinomas 23 3 per cent (males 18 0 per cent, females 32 9 per cent) and large cell carcinomas 14 8 per cent (males 13 2 per cent females 17 8 per cent) Other types constituted only 4 per cent The low number of epidermoid carcinomas among males and the high number of small cell anaplastic carcinomas in both sexes is unusual The majority of the tumours which could be localized were bronchial or central and only 12 per cent were peripheral Based on these histologically verified lung tumours the male/female ratio was found to be decreasing from 2 5 to 1 6 over the period If two 7 year periods were compared the rise in the incidence of lung tumours was found to be statistically significant for the combined tumour types I IV in both sexes and for adenocarcinomas in females. The ratio of Kreyberg Group I II has increased for males but remained stationary for females which throws doubt on the theory that the increase in female incidence can be attributed to smoking The WHO classification is a practical guide to the typing of lung tumours in routine diagnostic pathology The criteria are clear enough for the separation of the major histological types and all subtypes except those of small cell anaplastic carcinomas The incidence of lung carcinoma in Iceland is expected to continue to rise

The present investigation was undertaken to determine the distribution of the various histological types of carcinoma of the lung in Icelanders, based on the World Health Organization classification published in 1967 (12) Earlier reports using older classifications have indicated a high proportion of small cell anaplastic carcinomas and a low proportion of epidermoid carcinomas (5, 6, 23 24) The location of the tumours within the lungs was to be determined and correlated with the histological types

Only histologically verified tumours are included in the study From these an attempt is made to determine the frequency distribution of the histological types, the sex ratio and the age specific incidence within the period 1941–1968

An additional aim was to put the WHO classification on trial for its practical usefulness in a diagnostic laboratory

MATERIAL AND METHODS

The lung tumours were typed according to the WHO classification (12) (Table 1)

Most of the cases were obtained from the files of the Icelandic Cancer Registry which has reported cancer incidence since January 1 1955 The registry covers the whole population and with its wide range and careful selection one can be confident

Received 6 xii 72 Accepted 29 vi 73

Requests for reprints should be addressed to *Jónas Hallgrímsson* M D P O Box 150, Reykjavik Iceland

TABLE 1 *Histological Types of Lung Tumours in Iceland Based on the WHO Classification in the Period 1941–1968 by Sex and Percentage*

	Males		% of males	Females		% of females	Total	
	Number	% of type		Number	% of type		Number	% of total
I Epidermoid carcinomas	38	82 6	26 4	8	17 4	10 1	46	20 6
II Small cell anaplastic carcinomas	57	68 7	39 6	26	31 3	32 9	83	37 2
III Adenocarcinomas	26	50 0	18 0	26	50 0	32 9	52	23 3
IV Large cell carcinomas	19	57 6	13 2	14	42 4	17 8	33	14 8
VI Carcinoid tumours	2	28 6	1 4	5	71 4	6 3	7	3 1
X Sarcomas	*1*	*100 0*	*0 7*	*0*	*0*	*0*	*1*	*0 5*
XII Mesotheliomas	1	100 0	0 7	0	0	0	1	0 5
	144			79			223	

that all known cases have been included, whether diagnosed with histological examination or by other methods Lung tumours diagnosed before 1955 and previously reported by *Petersen* (19) were also included All histological examinations in the country have been and still are performed at the Department of Pathology of the University of Iceland and therefore the files of the institute both for surgical specimens and autopsies were checked and in that way a few as yet unlisted primary carcinomas of the lung were discovered The tumours which had been examined histologically were then selected for study and in most instances the slides and/or paraffin blocks were still available All the slides were stained with Haematoxylin Eosin stain In addition stains for mucin (Alcian green Alcian blue and PAS) and the combined staining method for keratin and mucin like substances (12) were used for identification of all the cases where the type was questioned on the H & E stain This included all the tumours initially classified as large cell carcinomas

Hospital records surgical reports and autopsy records were reviewed in order to determine the location of the primary tumours within the lungs This revision of the records helped in deciding whether the tumour was primary or secondary in the lung and a few cases were thus eliminated on account of insufficient evidence

RESULTS

Histological slides were available from 223 patients with primary tumours of the lung, diagnosed during the period 1941–1968

The results of the typing are presented in Table 1 A little over one third of the tumours are small cell anaplastic carcinomas and proportionally there is little difference between the sexes The second largest group is that of adenocarcinomas which are almost twice as common in females as in males Epidermoid carcinomas constitute a fifth of the tumours and most of them are in males The large cell carcinomas are nearly equally divided between males and females and thus relatively more frequent in females Only 9 tumours belong to the other histological types in the WHO classification No combination tumours were found and none of the tumours accepted into the study were considered to be unclassifiable

The morphological subtypes of types *II*, III and IV are shown in Table 2 These findings are dealt with in the discussion but it should be mentioned here that the majority of the large cell carcinomas are of subtype 1 which may be significant in relation to the possible common origin of that subtype and the adenocarcinomas

The tissue used for the histological examinations, as shown in Table *3*, came from the primary tumour in 211 out of 223 cases or 95 per cent An autopsy was performed in 152 instances or 68 per cent The histological typing is based on a metastatic tumour in

TABLE 2 *Carcinoma of Lung 1941–1968, Subtypes of Small Cell Anaplastic Carcinomas, Adenocarcinomas and Large Cell Carcinomas, by Sex*

	Subtype	Males	Females	Total
II Small cell anaplastic carcinomas	1	26	12	38
	2	10	6	16
	3	18	6	24
	4	3	2	5
III Adenocarcinomas	1a	21	22	43
	1b	4	3	7
	2	1	1	2
IV Large cell carcinomas	1	10	7	17
	2	5	3	8
	3	4	4	8
	4	0	0	0

only 5 per cent of the total cases and these were carefully scrutinized both clinically and radiologically before they were considered pulmonary in origin The metastases fell into the following types Epidermoid 2, small cell anaplastic 3, adeno 3 and large cell 4

The number of representative histological slides available was usually enough to be convincing for the tumour type involved That of course did not apply to the 36 tumours diagnosed by a single biopsy only, either from the primary tumour or a metastasis

The location of the tumours was determined from the autopsy and surgical reports No significant difference in incidence was found between the two sides There were 93 tumours in the upper lobes, 6 in the right middle lobe and 66 in the lower lobes A little over half of the epidermoid carcinomas were in the lower lobes, but all the other common types were more frequent in the upper lobes in both sexes The main and/or lobar bronchi were involved in 131 cases the segmental or smaller bronchi in 23 cases and 27 tumours were peripheral, i e had no visible connection with bronchi In 27 patients the main bronchus only was involved with tumour

The location according to the histological types was also determined Most of the epidermoid carcinomas or 40 out of 46 were in the larger or smaller bronchi Five epidermoid carcinomas were peripheral and none of them were considered to have originated in a scar One epidermoid carcinoma could not be localized The majority of the small cell anaplastic carcinomas were in bronchi or 62 out of 83 Four such tumours were only described as being centrally located There were 5 peripheral small cell anaplastic carcinomas and 2 of them had probably originated in scar tissue Twelve small cell anaplastic carcinomas could not be localized Twenty seven out of 52 adenocarcinomas were in bronchi and 2 were described as

TABLE 3 *Source of Tissue and Total Number of Specimens on Which the Study is Based*

	One source	Two or more sources	Total number
Autopsy	90	62	152
Resection	18	21	39
Bronchial biopsy	16	26	42
Lung biopsy	8	21	29
Metastasis	12	30	42

TABLE 4 *The Male/Female Ratio of Lung Tumours in Iceland by Types I–IV in Different Time Periods*

Types	1941–54		1955–61		1962 68		1955–68	
	Number	Ratio	Number	Ratio	Number	Ratio	Number	Ratio
I	2/0		13/1	13 1	23/7	3 1	36/8	4 5 1
II	8/3	2 7 1	19/11	1 7 1	30/12	2 5 1	49/23	2 1 1
III	5/3	1 7 1	9/5	1 8 1	12/18	0 7 1	21/23	0 9 1
IV	4/1	4 1	4/3	1 3 1	11/10	1 1 1	15/13	1 2 1
Total I–IV	19/7	2 7 1	45/20	2 2 1	76/47	1 6 1	121/67	1 8 1

being centrally located Peripheral adeno carcinomas totalled 9, and 4 of these had probably developed in scar tissue Fourteen tumours of this type could not be localized

Large cell carcinomas totalled 33 and 19 of them were in bronchi and 3 centrally located Peripheral large cell carcinomas totalled 7 and none of these were growing in scar tissue Four of the peripheral tumours contained mucin (subtype 1), 2 were without mucin (subtype 2) and 3 were of the giant cell type (subtype 3) Four large cell carci nomas could not be localized

Considering the tumours which could be localized, the proportion of each of the major histological types which was peripheral was *as follows epidermoid carcinomas* 11 per cent, small cell anaplastic carcinomas 6 per cent, adenocarcinomas 19 per cent and large cell carcinomas 21 per cent, an average of 12 per cent of the tumours being peripheral

Five of the bronchial adenomas (Type VI) were in the lobar bronchi of the left lung, one was in the right main bronchus and one was peripheral without a visible connection to a bronchus

The sex ratio of the tumours is shown in Table 4 There is a marked decrease in the male/female ratio over the years, when the tumour types I-IV are combined but it can be questioned whether the changes within each tumour type are meaningful on account of the low number of cases in each group The information on cases diagnosed before January 1, 1955 is less reliable than that on the later diagnosed cases since cancer regi stration started then, therefore a valid com parison between these periods may not be possible

In Table 5 the tumours are separated into Kreyberg Groups (Group I epidermoid and small cell anaplastic carcinomas and Group II *adenocarcinomas and carcinoid tumours)* and the ratios between the groups compared for several time periods There is a definite increase in the Group I/Group II ratio for males but for females the ratio remains fairly stationary *By definition (13) the large cell* carcinomas have to be eliminated from this comparison

In Tables 6 and 7 the age specific inci dence in both sexes is calculated for the

TABLE 5 *The Distribution of Lung Tumours in Iceland by Sex and Ratio of* Kreyberg *Groups I and II in Different Time Periods*

	Males		Females	
	Ratio	Number	Ratio	Number
1941–1954	1 7 1	10/6	0 8 1	3/4
1955–1961	3 2 1	32/10	1 7 1	12/7
1962–1968	4 4 1	53/12	1 1	19/20
1941–1968	3 4 1	95/28	1 1 1	34/31

TABLE 6 *Age Specific Incidence per 100 000 in Both Sexes for Combined Tumour Types I–IV in the Period 1955–1961*

	Age	Population	No of cases I	II	III	IV	Incidence I–IV
Males	25–34	12 153	1	0	0	0	8 23
	35–44	10 089	0	2	2	0	39 65
	45–54	8 246	3	7	0	2	145 53
	55 64	6 424	6	7	3	1	264 63
	65–74	3 973	1	0	4	1	151 02
	75 and older	1 956	2	3	0	0	255 62
Total			13	19	9	4	
Females	25–34	11 624	0	0	0	0	
	35–44	9 733	0	2	0	0	20 55
	45–54	8 057	0	0	0	0	
	55 64	6 613	1	3	2	1	105 85
	65–74	4 473	0	3	1	2	134 14
	75 and older	2 846	0	3	2	0	175 69
Total			1	11	5	3	

combined tumour types I-IV in the two 7-year-periods 1955–1961 and 1962–1968, i e the first 14 years during which the Cancer Registry has been functioning The comparison shows a marked rise in the incidence in both sexes for the age groups 45 and older and a fall in the males 75 and older

Chi-square tests (17) on the difference in incidence between the two periods were applied for each tumour type by sex and 10-year age groups The results for adenocarcinoma in females was found to be significant to the 5 per cent level but insignificant for the other types in females and all types in

TABLE 7 *Age Specific Incidence per 100 000 in Both Sexes for Combined Tumour Types I–IV in the Period 1962–1968*

	Age	Population	No of cases I	II	III	IV	Incidence I IV
Males	25–34	11 937	0	1	0	0	8 38
	35–44	11 334	0	0	1	1	17 65
	45–54	9 127	11	9	2	3	273 91
	55 64	7 161	7	11	4	4	363 08
	65–74	4 945	4	7	4	3	364 00
	75 and older	2 441	1	2	1	0	163 87
Total			23	30	12	11	
Females	25–34	11 508	0	0	0	0	
	35–44	10 817	0	0	1	0	9 24
	45–54	9 007	2	4	1	4	122 13
	55–64	7 224	2	5	6	1	193 80
	65–74	5 493	2	2	3	2	163 84
	75 and older	3 289	1	1	7	3	364 85
Total			7	12	18	10	

males When chi square tests were applied for the combined tumour types I-IV with subgroups of sex and 10-year age groups the results were found to be significant to the 5 per cent level for males and significant to the 1 per cent level for females By the same method the results were insignificant in both sexes if the tumours were arranged in Kreyberg groups

The mean age of the patients at the time of histological diagnosis was calculated for types I-IV in both sexes, being 58 3 years for males and 63 5 years for females This difference in age is statistically significant (1 per cent significance) In each sex the mean age was slightly higher for the adenocarcinoma group than for the other type groups, but the difference was not statistically significant (5 per cent significance) The mean age for the four types I-IV combined was also calculated in each of the two periods 1955–1961 and 1962–1968 and no significant age difference was found for either sex

DISCUSSION

In Iceland carcinoma of the lung is the third commonest malignant tumour in males (6 7 per cent) and the ninth in females (3 3 per cent), according to the Icelandic Cancer Registry (21) which lists all malignant tumours diagnosed clinically and histologically The majority of the registered tumours are confirmed by histological examination either from surgical specimens or at autopsy In the cases of lung tumours, 286 cases were registered in the period 1955–1968 and 195 of them or 73 3 per cent included at least one tissue examination Most of these are included in this study During these years the autopsy rate at the larger hospitals in Reykjavik has remained at a high level and with a few exceptions all the patients who have died at the university hospital, Landspitalinn, have come to autopsy The only thoracic surgical unit in the country was established at Landspitalinn in 1955 and therefore a large number of these patients had been referred there at one time or the other for diagnostic service, surgery or terminal care During the last 14 years of the study period there has therefore not been any significant change in facilities or attitude towards the need for tissue examination in the cases of malignant tumours. With this in mind it was considered justifiable to calculate the incidence rates of these tumours from the histologically verified cases only In a previous study (24) on the incidence of lung carcinoma in Iceland, based on all cases registered, a rise comparable to the one documented here has been found

The number of tumours presented here is small but yet it represents the total incidence in the population The standard of living in Iceland is high and modern, the public health system well organized and the documentation of disease is good and therefore it is hoped that this small series of cases may serve for comparative studies with series coming from larger populations in other countries

This investigation is based on the opinion of one pathologist although he has consulted his colleagues about the most debatable cases This may be open to criticism On the other hand a single investigator is likely to apply criteria such as those set forth by the WHO classification in a more uniform and reproducible manner than he and his colleagues would do in their routine work Before

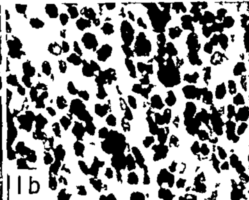
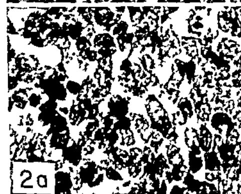
---

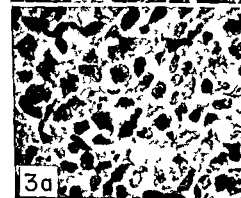
Fig 1 (1076/53) A bronchial biopsy from a small cell anaplastic carcinoma, showing both the fusiform cell type (a) and the lymphocyte like cell type (b) (H + E) × 150

Fig 2 (1301/61 and s 128/61) Small cell anaplastic carcinoma A bronchial biopsy (a) shows round cells of uncertain classification but sections from the autopsy (b) show predominantly fusiform cells but round cells are also present (H + E) × 150

cell
(a)
from

the subcutis taken one year later was classified as fusiform cell type (b) and in the autopsy the sections showed both fusiform cells and lymphocyte like cells (c) (H + E) × 150


1a

1b

2a

2b

3a

3b

3c

comparison can be made between the results of studies like the one presented here, considerations should be given to the interobserver variation known to exist in the histological classification of lung tumours (7, 29, 32) A panel of the investigators involved could be convened to find out whether serious differences in the application of the criteria exist and in that event formulate methods by which these difficulties could be overcome

In a recent article on lung cancer, *Anderson* (1) has this to say on statistical studies of bronchial carcinoma "Until general agreement about diagnostic criteria and terminology is reached, and until they are actually used, comparison of much published data will have little meaning" The WHO classification is probably the closest answer yet to this, although it may not offer the solution to all the problems and incorporate all the personal opinions experienced pathologists in the field of cancer may have

In the chapter on the general principles of lung tumour typing in the WHO classification guide (12) there is much optimism regarding the ease of typing provided the specimens are well preserved and the diagnostic criteria closely followed The conclusion drawn from the present investigation is that the WHO classification is a good and practical guide for typing the most common varieties of lung tumours, i e the main types and subtypes of III and IV, whereas the subtypes of the small cell anaplastic carcinomas (type II) are difficult to separate

In a recent article, *Kreyberg* (14) has indeed pointed out that, in the ordinary routine examination of material presented for diagnosis, it may be impossible to distinguish between the different subtypes of small cell anaplastic carcinomas In reviewing the Icelandic material some of the reasons for these difficulties have emerged In the first place, the method of obtaining surgical tissue biopsies may create histological artefacts such as the commonly seen squeezing by the biopsy forceps Secondly the tissues are in different conditions upon arrival at the pathology laboratory, surgical specimens arrive fresh or have been immediately fixed following removal, but cadavers are at different stages of post-mortem autolysis Thirdly, tissue sections and paraffin blocks deteriorate with time unless properly stored under optimal conditions of light, temperature and humidity and fine histological details may thus be lost Fourthly, the small cell anaplastic carcinomas sometimes are composed of more than one type of cells In some cases even the 3 most commonly recognized subtypes may all be present at the same site or at different sites, at the same time or at different times, during the course of the disease One cannot always decide between an artefact and true pleomorphism of cells When these problems came up in the study, either the subtype seen in the best preserved tissue or the one most commonly encountered in the available sections was chosen Figs 1, 2 and 3 illustrate the problem and need no further comments here Other investigators have also emphasized the variable histological appearance of small cell anaplastic carcinomas (2, 3, 30)

In a retrospective study like the one presented here, certain technical difficulties are involved regarding the staining of all the tumours with the recommended combined stain for keratin and mucin like substances It was therefore decided to limit the recutting of available old blocks for this particular stain to the tumours which did not obviously conform to the criteria set forth, i e contain keratin, form glandular spaces or the typical picture for small cell anaplastic carcinoma It is hoped that the value of the study has not suffered significantly by this

Whenever incidence data are to be compared there must be conformity not merely in the use of criteria and terminology, but the source of the material must also be similar It is well known that the distribution of tumour types differs greatly between surgical and autopsy specimens (27) A rather recent study based on both surgical and autopsy material, as the Icelandic one is, is the one by *Uys* from Cape Town (25) who also uses the new WHO classification

In Iceland the commonest tumour type among males is the small cell anaplastic carcinoma (39 6 per cent) followed by epidermoid carcinoma (26 4 per cent), adenocarcinoma (18 0 per cent) and large cell carcinoma (13 2 per cent) In the white population of Cape Town the order is that of epidermoid carcinoma (44 3 per cent) small cell anaplastic carcinoma (28 3 per cent), large cell carcinoma (21 5 per cent) and adenocarcinoma (5 8 per cent)

In females in Iceland the commonest types are small cell anaplastic carcinoma and adenocarcinoma (each 32 9 per cent) followed by large cell carcinoma (17 8 per cent) and epidermoid carcinoma (10 1 per cent) In the white females of Cape Town the order is that of small cell anaplastic carcinoma (34 0 per cent) large cell carcinoma (25 5 per cent) epidermoid carcinoma (23 4 per cent) and adenocarcinoma (17 0 per cent)

Studies by *Kreyberg* & *Saxen* (10) and *Kreyberg* (11, 13) are based on the same classification but surgical specimens constitued the largest part of their material In Finland the order of frequency among males was that of epidermoid carcinoma (55 8 per cent), small cell anaplastic carcinoma (25 5 per cent) and adenocarcinoma (6 4 per cent) and among females that of adenocarcinoma (48 1 per cent), small cell anaplastic carcinoma (18 5 per cent) and epidermoid carcinoma (7 4 per cent) In Norway the order for males was that of epidermoid carcinoma (62 0 per cent) small cell anaplastic carcinoma (16 1 per cent) and adenocarcinoma (13 5 per cent) and for females that of adenocarcinoma including bronchiolo-alveolar carcinoma (58 1 per cent), epidermoid carcinoma (11 3 per cent) and small cell anaplastic carcinoma (7 3 per cent) These results differ in several ways from those pre

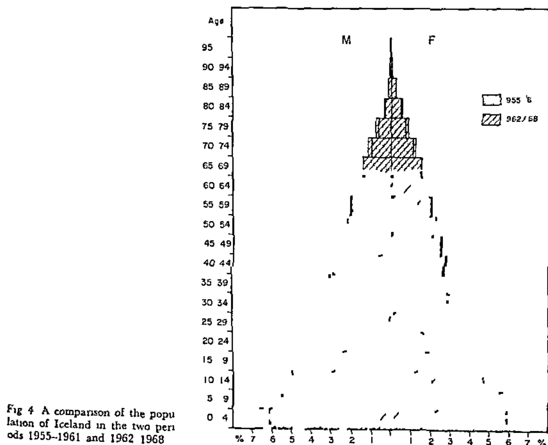


Fig 4 A comparison of the population of Iceland in the two periods 1955–1961 and 1962 1968

sented here, for example in the frequency of large cell carcinoma which is less than 3 per cent in Finland and Norway (11) The biological significance of these differences is doubtful since in the Icelandic series the proportion of surgical specimens is smaller and the application of the classification may differ

The sex distribution of carcinoma of the lung in Iceland (Table 4) is different from that reported in other countries, the male/female ratio of the histologically verified cases between 1941 and 1968 being 1 8 and for the period 1962–1968 it is 1 6 In Cape Town (25) it is 6 5 and in other recent investigations (13, 26, 31) the males outnumber the females many times According to *Spencer* (22) this tumour was almost equally common in both sexes 50 years ago, but from then on the proportion of male cases has risen continuously until recently when the difference narrowed somewhat *Lombard* (16) has seen the same trend and thinks that cigarette smoking among females may be important as an a etiological factor Smoking among Icelanders of both sexes is growing and most of the patients with WHO types I and II carcinoma have smoked (23, 24) Among males the ratio of *Kreyberg* Group I II tumours is certainly rising whereas among females the ratio has remained more stationary (Table 5) In Norway the Group I II ratio has also increased for males but for females it has decreased (13) On the assumption that smoking mainly induces Group I tumours and other causative factors lie behind Group II tumours, the ratio for males is confirmatory but for females one has to be cautious in associating the high female incidence with smoking The highest rise of any one type of tumour was that of adenocarcinoma in females which by most investigators is not thought to be induced by tobacco smoke *Haenszel* (9) has emphasized the ethnic variation in the sex distribution of tumours and this may be a factor in Iceland The rising female incidence of lung tumours is not observed everywhere, *Byrd* (4) for instance reports a constant average annual incidence for females in Olmsted County, Minnesota during 1935–1964 while for males it increased eightfold in the same period

The distribution of the Icelandic tumours over bronchial and peripheral sites is about the same as that reported by *Liebow* (15) who found that about 12 per cent of the tumours were peripheral. Many authors (8, 18, 22, 28) have indicated that the origin of lung tumours probably is more often in the peripheral part of the lungs than is apparent at the time of pathological examination As the present evaluation of the tumour location was retrospective and based on written reports, no opinion of their exact origins can be expressed

The age distribution in Iceland is the same as elsewhere including a slightly higher mean age for females (22) *Bryson* & *Spencer* (3) found no significant difference in the mean age of the patients when they were grouped according to tumour types and our data confirm this

The incidence of lung tumours in Iceland has been rising steadily (Tables 6 and 7) Can we expect the same continuous rise in the future? At present we do not have the answer, but it may be predicted that the relative incidence and also the actual number of tumours will increase on account of the growing habit of cigarette smoking and the continued population increase The age distribution of the population of Iceland for the two periods 1955 1961 and 1962–1968 is shown in Fig 4 The growth in the second period was mostly in the age groups 5–20 in both sexes Considering the low infant mortality and the long life expectancy in Iceland a major rise in the number of lung tumours may be expected within 20 to 30 years from now

---

The author is grateful to *Hrafn Tulinius* M D IARC, Lyon and Dr *Helgi Sigvaldason*, Reykjavik for their advice

REFERENCES

1 *Anderson, W A D* Contributions of pathology to problems of human and experimental lung cancer Am J Clin Path *46* 3–26, 1966

2 *Azzopardi, J G* Oat cell carcinoma of the bronchus J Path Bact *78* 513–519, 1959

3 *Bryson, C C & Spencer, H* Carcinoma of the bronchus Quart J Med N S *20* 173–187, 1951

4 *Byrd, R B, Nobrega, F T, Diverttie, M B, Carr, D T, Woolner, L B & Kurland, L T* The 30 year trend of bronchogenic carcinoma in Olmsted County, Minnesota, 1935–1964 J Chronic Dis *24* 9–18, 1971

5 *Dungal, N* Cancer in Iceland In Raven, R W (Ed) Cancer, 1 ed Vol 3, Butterworth and Co Ltd, London, 1958, p 270–271

6 *Dungal, N* Lung cancer in Iceland Lancet *245* 1350–1352, 1961

7 *Feinstein, A R, Gelfman, N A & Yesner, R* with the collaboration of *Auerbach, O, Hackel, D B & Pratt, P C* Observer variability in the histopathologic diagnosis of lung cancer Am. Rev Resp Dis *101* 671–684, 1970

8 *Garland, L H, Beier, R L, Coulson, W, Heald, J H & Stein, R L* The apparent sites of origin of carcinomas of the lung Radiology *78* 1–11, 1962

9 *Haenszel, W* Cancer mortality among the foreign-born *in* the United States. *J Nat* Cancer Inst *26* 37–132, 1961

10 *Kreyberg, L & Saxén, E* A comparison of lung tumor types in Finland and Norway Brit J Cancer *15* 211 214, 1961

11 *Kreyberg, L* Histological lung cancer types A morphological and biological correlation Acta Path Microbiol Scand Suppl *157*, 1962 p 30

12 *Kreyberg, L, Liebow, A A & Uehlinger, E A* Histological typing of lung tumours Inter national histological classification of tumors No 1 World Health Organization, Geneva, 1967

13 *Kreyberg, L* Aetiology of lung cancer A morphological epidemiological and experi mental analysis. 1 ed Universitetsforlaget Oslo-Bergen Tromsö, 1969 pp 19–24

14 *Kreyberg, L* Comments on the histological typing of lung tumors Acta Path Microbiol Scand Section A *79* 409–422, 1971

15 *Liebow, A A* Tumors of the lower respira tory tract In Atlas of Tumor Pathology, Sec tion V Fascicle 17 1 ed Armed Forces Insti tute of Pathology, Washington D C 1952 pp 17 and 67

16 *Lombard, H L & Huyck, E P* An epidemiological study of lung cancer among females. Growth *32* 41–56, 1968

17 *Mantel, N & Haenszel, W* Statistical aspects of the analysis of data from retrospective studies of disease J Nat Cancer Inst *22* 719–748, 1959

18 *Meyer, E C & Liebow, A A* Relationship of interstitial pneumonia honeycombing and [illegible]

19 [illegible]

20 [illegible] *C* The duration of carcinoma of the lung Dis Chest *23* 50–71, 1953

21 *Ringertz, N* (Ed) Cancer incidence in Fin land, Iceland, Norway and Sweden Acta path *Microbiol Scand Section A, Suppl 224*, 1971 p 12

22 *Spencer, H* Pathology of the lung 2 ed Pergamon Press Ltd, London 1968 pp 805–806

23 *Thorarinsson, H, Hallgrimsson, J, Bjarnason, Ó & Petersen, G* Carcinoma of the lung in Iceland Dis. Chest *52* 754–759, 1967

24 *Thorarinsson, H* Carcinoma of the lung in Iceland Scand J Thor Cardiovasc. Surg *3* 31–38, 1969

25 *Uys, C J Bronchial carcinoma in the popu*lation groups of Cape Town South African Cancer Bulletin *14* 8–14, 1970

26 *Vincent, T N, Satterfield, J V & Ackerman, L V* Carcinoma of the lung in women Cancer *18* 559–570, 1965

27 *Walter, J B & Pryce, D M* The histology of lung cancer Thorax *10* 107–116, 1955

28 *Walter, J B & Pryce, D M* The site of origin of lung cancer and its relation to histological type Thorax *10* 117–126, 1955

29 *Weiss, W Boucot, K R & Cooper, D A* The histopathology of bronchogenic carcinoma *and its relation to growth rate, metastasis and* prognosis Cancer *26* 965–970, 1970

30 *Willis, R A* The incidence and histological types of pulmonary carcinoma, with comments on some fallacies and uncertainties Med J Australia *I 48th year* 433–440, 1961

31 *Wynder, E L, Mabuchi, K & Beattie, E J* The epidemiology of lung cancer Recent trends JAMA *213* 2221–2228 1970

32 *Yesner, R, Gerstl, B & Auerbach, O* Application of the World Health Organization classification of lung carcinoma to biopsy material. Ann Thorac Surg *1* 33–49 1965

Acta path microbiol scand Section A 81, 824–830, 1973

COMPARISON BETWEEN MICROSCOPICAL METHODS AND CULTIVATION FOR DEMONSTRATION OF TUBERCLE BACILLI IN EXPERIMENTAL TUBERCULOUS INFECTION

B WINBLAD and M DUCHEK

Departments of Pathology and Urology, University of Umeå, Umeå, Sweden

As part of an experimental study of the routes of spread of urogenital tuberculosis, in which guinea pigs were inoculated with *Mycobacterium tuberculosis* ($H_{37}Rv$), the efficacy of a standard staining method (haematoxylin eosin), specific staining for tubercle bacilli (Ziehl-Neelsen and fluorescent staining) and culture for the demonstration of tuberculous infection were compared By fluorescence microscopy of sections stained with auramine rhodamine, tubercle bacilli were more clearly seen if epi illumination was used instead of trans illumination at higher magnifications Fluorescence microscopy gave a clearly larger pro portion of positive findings than did the routine Ziehl Neelsen technique, and the time required for examination was considerably reduced Detection by fluorescence microscopy was generally equivalent to detection by culture

Tuberculous infection cannot be adequately evaluated by means of macroscopic observation and routine histological examination alone The presence of tubercle bacilli must be demonstrated Culture of specimens from different organs and body fluids is an effective procedure to demonstrate the bacteria If, however, the problem is to study the *routes of spread of the infection*, microscopical methods are more accurate than culture

As a preliminary step in an experimental investigation of the spread of infection with *Mycobacterium tuberculosis* in the urogenital tract, the relative value of different micro scopical methods and cultivation for demonstration of tuberculous infection was studied

Many methods of staining tubercle bacilli have been described, but one of the earliest, the Ziehl-Neelsen technique, is still widely used with good results *Hagemann* (1937) was the first to report the advantages of a fluorescent dye Fluorescent staining has proved satisfactory for the detection of tubercle bacilli *in sputa* (*Richter & Halova* 1968, *Parrot et al* 1970, and others) and has given promising results when applied to histological sections (*Gray* 1953, *Kuper & May* 1960, *Braunstein & Adriano* 1961, *Yamaguchi & Braunstein* 1965, *Richter* 1969)

Received 3 iv 73 Accepted 6 vi 73

Requests for reprints should be addressed to Dr *B Winblad*, Institute of Pathology University of Umeå, S 901 87 Umeå Sweden

MATERIAL AND METHODS

0 05 ml of a14 day-old culture of a human strain of tubercle bacilli ($H_{37}Rv$), containing 15 000-75,000 bacteria per ml was inoculated by a dispos

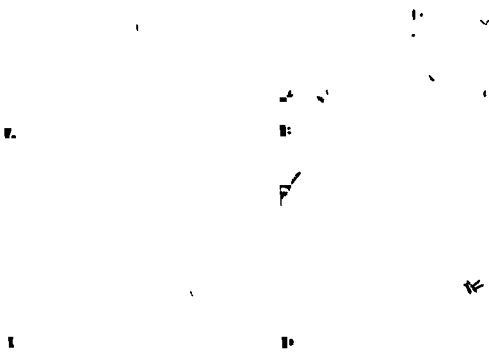


Fig 1 Tubercle bacilli in section of epididymis. Auramine rhodamine fluorescent stain Barrier filter 50 a Transillumination × 400 b Epi illumination × 400 c Transillumination × 800 d Epi illumination × 800

able tuberculin syringe into the epididymis or seminal vesicle on one side in 31 sexually mature guinea pigs (*Duchek & Winblad* 1973)

The animals were killed and sectioned after time intervals ranging from 3 to 5 weeks

At section both testes epididymes the prostate the seminal vesicles and the kidneys were removed from each animal One half of each organ was used for culture the other half for microscopic studies Each organ was dissected using newly sterilized instruments

Cultivation

Culture was performed on Lowenstein Jensen s medium after mincing of the tissues Three cultures were taken from each organ and culture was judged to be positive if one of these showed bacterial growth after incubation for 6 weeks

Histological Technique

The organs were fixed in neutral 10 per cent formalin solution Specimens were embedded in paraffin and cut into serial sections about 4 μ thick Two sections were mounted on each of three slides for staining with haematoxylin eosin Ziehl Neelsen stain and fluorescent staining Staining according to Ziehl Neelsen was carried out according to the procedure outlined in Handbook of Bacteriological Technique (*Baker* 1962)

The auramine rhodamine fluorescent stain for acid fast bacilli in histological sections was based on the method described by *Mattheei (1950)* with the addition of potassium permanganate as counter stain (*Kuper & May* 1960) The auramine rhodamine stain was freshly made up for each group

Microscopical evaluations were performed blind by one of us (B W) that is without knowledge of the results of other staining methods or of culture The examination time for each of the two sections stained with Ziehl Neelsen and fluorescent stain respectively was limited to 30 minutes

Optical Equipment

Sections stained with haematoxylin eosin or with Ziehl Neelsen stain were examined using a Zeiss Standard RA microscope with 10/0 22 40/0 65

TABLE 1 *Comparative Efficacy of the Methods Used for the Detection of Tuberculous Infection*

Group*	H E†	Z N§	A R§	Culture$	Number of organ pieces	Per cent of total
1	+	+	+	+	33	15 2
2	+	—	+	+	20	9 2
3	+	—	+	—	9	4 1
4	—	—	—	+	8	3 7
5	+	+	+	—	6	2 8
6	—	—	+	—	5	2 3
7	+	—	—	+	3	1 4
8	—	—	+	+	3	1 4
9	+	—	—	—	2	0 9
10	—	—	—	—	128	59 0

* Group 1-10 shows different combinations of results obtained by staining methods and cultivation
† In the H E (haematoxylin eosin stain) + denotes granulomatous inflammation, – denotes its absence
§ In the Z N (Ziehl Neelsen stain) and A R (auramine rhodamine fluorescent stain), + and – denote the presence or absence of tubercle bacilli in the tissue sections
$ + and – denote positive or negative cultures

and 100/1 25 (oil) objectives and 12 5 × binocular eye pieces

For fluorescence microscopy, a Zeiss Large Universal Fluorescence Microscope equipped with a super pressure mercury lamp HBO 200 W was used, together with an exciter filter BG 12/3 which completely absorbs red light As barrier filters 47 and 50 proved most useful The examinations were carried out in a room with subdued lighting A Neofluar 16/0 40 objective was used for fluorescent scanning, and a Neofluar 40/0 75 objective to determine the morphology Oil immersion 100/1 25 was used for photomicrography 12 5 × binocular eyepieces were used throughout The specimens were examined in both transmitted light and epi illumination

The bacteria were seen more clearly at higher magnifications if epi illumination (Figs. 1b and d) was used instead of transillumination (Figs 1a and c)

Photomicrography

A C 35M camera with automatic shutter was used The film was Agfachrome 50 L Exposure time varied from 15 seconds to 10 minutes

RESULTS

The results of various microscopical methods and of culture of a total of 217 organ pieces from the 31 infected guinea pigs are summarized in Table 1 All the combinations of results obtained are given, in the order of the percentage of the total number of observations they comprise

It appears from Table 1 that H-E staining failed to demonstrate any granulomatous changes in a total of 16 specimens (7 4 per cent) which were positive for bacilli according to another staining technique or on culture (groups 4 6 and 8) Granulomatous changes were seen in only 2 specimens (group 9) which were negative in other staining methods and on culture

In groups 2, 3, 6 and 8, Z-N staining was negative, but fluorescence microscopy demonstrated bacilli in 37 specimens (17 0 per cent) Culture was negative for 14 of these specimens No specimen was negative in fluorescence microscopy but positive to Z N staining There were 6 specimens with positive Z N staining (as well as positive H E staining and fluorescent staining) that were negative on culture (group 5) Positive culture but negative Z-N staining (groups 2, 4, 7 and 8) was encountered for 34 specimens among which 23 were positive to fluorescent staining

Twenty specimens showed positive fluorescent staining and negative cultures (groups 3, 5 and 6), and 11 (groups 4 and 7) were positive on culture although fluores

cent staining and Z N staining were negative

It also appears from Table 1 that it applies to no less than 59 per cent of the specimens that all examinations were negative (group 10) This is a result of the relatively short period of infection, chosen in order that infection would be limited to the urogenital tract The investigation included the genital organs from the side contralateral to that on which infection was introduced, as well as the kidneys which in the guinea pig are rather resistant to tuberculous infection (*Rich* 1944)

An analysis of some of the discordant groups in Table 1 shows that certain differences in the results were related to the site of inoculation Among the 37 specimens positive in fluorescence microscopy and negative to Z N staining, 13 were from prostatic tissue In 6 of these cases only a few bacteria were demonstrated by the fluorescent technique and cultures were negative, in the remaining 7, no more than 5 colonies grew out in any of the three tubes inoculated with material from each organ piece Ten of the specimens that were negative to Z-N staining and positive in fluorescence originated from seminal vesicles in which, although they were the site of injection and clear granulomatous changes were seen in the H-E stained preparations, only a few bacteria were demonstrated by fluorescent staining Cultures of these specimens were positive, with an average of 20 colonies per tube If injection was applied into the epididymis, there was only one specimen that was negative to Z-N staining, positive in fluorescence and clearly positive in culture

Analysis of the 6 specimens with positive microscopical staining reactions but negative on culture (group 5) shows that 3 originated from testes epididymes of animals in which injection was applied into the seminal vesicle and 3 from prostates of animals in which injection was applied to the seminal vesicle Specimens which were positive only in fluorescence microscopy and negative in the other microscopical methods and on culture (group 6) were of similar origin

In the 11 specimens that were positive on culture but negative in fluorescence, bacterial growth on culture was usually slight, in general no more than 3 colonies per tube

Comparison of the two specific staining methods used for the demonstration of tubercle bacilli showed that fluorescence microscopy is superior to the Z-N method The examination of the slides is easier and more rapid The fluorescent rods were often immediately identifiable Using the A R combination, the acid-fast bacilli were seen in various colours depending on the type of barrier filter The barrier filters 47 and 50 were most useful, giving a blue and a dark green background, respectively Using barrier filter 50, the bacteria were easier to localize in relation to tissue components The tubercle bacilli were more easily identified if epi-illumination was used instead of transillumination at higher magnifications If screening involved an objective magnification of × 16, fluorescence was somewhat more intense if transillumination was used

DISCUSSION

Most of the comparisons between different staining methods and culture for the demonstration of tubercle bacilli have been performed on sputum A comparison between the published results obtained in various laboratories using a variety of techniques lies in the uncertainty that the groups of specimens under review are strictly comparable (*Clegg & Foster-Carter* 1946)

In some early investigations, the fluorescence technique was not more sensitive than the Z N staining (*Didion* 1939, *Freimann & Mokotoff* 1943, *Lind* 1949) The development of better equipment and modern dye combinations have, however, increased the sensitivity of the fluorescence method over the past 20 years By now, it regularly detects a percentage of positive samples greater than that obtained by Z N staining (*Truant et al* 1962, *Richter & Halova* 1968, *Parrot et al* 1970) All microscopic methods, includ-

ing the fluorescence technique, have been reported to be clearly inferior to culture (*Didion* 1939, *Freimann & Mokotoff* 1943, *Holm & Plum 1943*, *Clegg & Foster Carter* 1946, *Needham* 1957) *Holm & Plum* (1943), for example, found about 50 per cent more positive sputa by culture than by Z-N staining or fluorescence microscopy However, in a more recent investigation, a quantitative comparative study of 25 sputa with strongly positive findings, *Richter & Halova* (1968) found fluorescent staining to be the most sensitive means of detection, demonstration by culture being next best, and the classical Z N staining technique to be least sensitive

Bachmann & Finke (1939) were the first to apply fluorescent staining to tissue sections, and were thereby able to demonstrate greater numbers of tubercle bacilli in tissue than otherwise possible in Z N stained sections They also pointed out the superiority of the fluorescence method for the detection of scattered bacteria in tissue sections *Gray* (1953) stated that the fluorescence method reveals even five times the number of organisms otherwise revealed by the Z N technique in adjacent sections from a block of tissues Other comparisons between staining methods applied to tissue sections and culture (*Kuper & May* 1960, *Braunstein & Adriano* 1961, *Truant et al* 1962, *Koch & Cote* 1965, *Yamaguchi & Braunstein* 1965, *Richter* 1969), mainly carried out on human material, have yielded similar results

If, however, the fluorescent technique is applied to such specimens its specifity may be doubted *Wen Lan Lou Wang* (1969) reported that the appearance of acid fast bacilli in many of the sections could be due to contamination of the paraffin by non specific acid fast bacilli He suggested that these contaminants may multiply in the paraffin and be present in the paraffin blocks from which tissue sections are made

We have tried to maintain control over specificity, using a single well defined strain, H_3 Rv, in our experimental studies in guinea pigs The risk of confusion with atypical acid fast bacilli would seem to be virtually nil The work was conducted under strictly aseptic conditions, using newly sterilized instruments for each organ at operation, autopsy and in preparation of material for culture New paraffin was always used for embedding A paraffin dispenser or Autotechnicon was not used

Under these experimental conditions we have been able to confirm the superiority of the fluorescence method over the classical Ziehl-Neelsen method for the detection of tubercle bacilli Not only the method has a higher accuracy but the staining technique is simpler and the microscopy more rapid Above all, the fluorescent technique was valuable if applied to organs in which only a few bacteria were present, this has previously been pointed out by *Bachmann & Finke* (1939), *Haebler & Murray* (1954) and by *Parrot et al* (1970) The great reduction in the time required for examination, compared with that required for Z N stained sections, was a major advantage. *Larsen* (1940) estimated, using fluorescent technique, that it was possible to examine a 10 times larger area in less than 1/3 the time required for examination of Z-N stained specimens The brilliance of the bacteria makes it possible to identify them at a lower magnification, the use of oil immersion is not necessary

The high frequency of negative Z-N staining in spite of positive fluorescence microscopy (37 specimens) is probably explained in part by the fact that we limited our inspection to 30 minutes A thorough examination of Z-N stained specimens under oil immersion is time-consuming, requiring up to 8 hours per specimen, according to some authors (*Bogen* 1941)

The most striking features of bacteria examined by the fluorescence technique are their brilliance and the clarity with which their morphology is demonstrated which make it possible to identify them at a lower magnification, the use of oil immersion is not necessary These qualities make it easy to distinguish the bacteria from artefacts The fluorescence of artefacts is usually less and

yellowish in colour, and artefacts are often larger and irregular (*Wellman* 1962).

Disadvantages such as a certain amount of difficulty in focusing (*Freimann & Mokotoff* 1943) and of orientation in tissue sections stained with fluorescent dyes can largely be avoided by adequate counterstaining, and are eliminated if epi illumination is used. Using a low magnification objective with normal aperture, fluorescence is often more intense if trans illumination is used. Using a higher magnification objective, epi-illumination gives greater brilliance and lower background fluorescence. Under epi-illumination the objective serves as its own condensor and the illumination of the field is as uniform as possible. Since only the observed field is illuminated, other parts of the section are not subjected to fading.

Failure to demonstrate bacteria by culture cannot be accepted as conclusive evidence of their absence. The limited volume of the samples used for culture in this study may certainly play some part. For example, it is difficult to section prostatic tissue into representative pieces, and sectioning of the testis epididymis sometimes reveals only isolated granulomas in the lymphatics and surrounding membranes. Under our experimental conditions it is not probable that falsely negative cultures would result from non viable organisms or technical errors.

It should be made clear that no single laboratory method for the diagnosis of tuberculosis is infallible. Our results show a range of positive and negative findings obtained by both microscopic and cultural procedures. It should be stressed that neither clinical nor experimental investigations should be based on one method only.

The results of this study made it clear that, in further experimental work on the routes of spread of urogenital tuberculosis, histological examination of sections stained with haematoxylin-eosin, in combination with fluorescent staining with auramine-rhodamine for the demonstration of tubercle bacilli, would be acceptable for mapping the spread of experimental infection.

This work was supported by grants from the *Medical Faculty, University of Umeå*, Umeå, Sweden.

REFERENCES

1. *Bachmann, W. & Finke, L.*: Über die fluoreszenzmikroskopische Darstellung von Tuberkelbazillen im Gewebe. Dt. Med. Wschr. *38*: 1474–1475, 1939.
2. *Baker, F. J.*: Handbook of bacteriologic technique. Butterworth, London, 1962, pp. 31–32.
3. *Bogen, E.*: Detection of tubercle bacilli by fluorescence microscopy. Am. Rev. Tuberc. *44*: 267–271, 1941.
4. *Braunstein, H. & Adriano, S. M.*: Fluorescent stain for tubercle bacilli in histologic sections. I. Diagnostic efficiency in granulomatous lesions of lymph nodes. Am. J. Clin. Path. *36*: 37–40, 1961.
5. *Clegg, J. W. & Foster Carter, A. F.*: Detection of tubercle bacilli by the fluorescence technique. Br. J. Tuberc. *40*: 98–105, 1946.
6. *Didion, H.*: Über den fluoreszenzmikroskopischen Nachweis von Tuberkelbakterien. Klin. Wschr. *40*: 1315–1318, 1939.
7. *Duchek, M. & Winblad, B.*: An experimental method for studying the spread of genital tuberculosis. Urol. Research *1*: 32–36, 1973.
8. *Freimann, D. G. & Mokotoff, G. F.*: Demonstration of tubercle bacilli by fluorescence microscopy. A comparative evaluation of the fluorescence and Ziehl Neelsen methods in the routine examination of smears for acid fast bacilli. Am. Rev. Tuberc. *48*: 435–442, 1943.
9. *Gray, D. F.*: Detection of small numbers of mycobacteria in sections by fluorescence microscopy. Am. Rev. Tuberc. *68*: 82–95, 1953.
10. *von Haebler, T. & Murray, J. F.*: Fluorescence microscopy as a routine method for the detection of m. tuberculosis and m. leprae. S. Afr. Med. J. *28*: 45–48, 1954.
11. *Hagemann, P. K. H.*: Fluoreszenzmikroskopische Untersuchung über Virus- und andere Mikroben. Zbl. Bakt., Abt. Orig. *140*: 184–187, 1937.
12. *Holm, J. & Plum, N.*: On the demonstration of tubercle bacilli in the sputum in man. Comparison between fluorescence microscopy, Ziehl Neelsen microscopy and cultivation. Acta Tuberc. Scand. *17*: 13–36, 1943.
13. *Koch, M. L. & Cote, R. A.*: Comparison of fluorescence microscopy with Ziehl Neelsen stain for demonstration of acid fast bacilli in smear preparations and tissue sections. Am. Resp. Dis. *91*: 283–284, 1965.
14. *Kuper, S. W. A. & May, J. R.*: Detection of acid fast organisms in tissue sections by fluo-

ing the fluorescence technique, have been reported to be clearly inferior to culture (*Didion* 1939, *Freimann* & *Mokotoff* 1943, *Holm* & *Plum 1943*, *Clegg* & *Foster-Carter* 1946, *Needham* 1957) *Holm* & *Plum* (1943), for example, found about 50 per cent more positive sputa by culture than by Z-N *staining or fluorescence microscopy* However, in a more recent investigation, a quantitative comparative study of 25 sputa with strongly positive findings, *Richter* & *Halová* (1968) found fluorescent staining to be the most sensitive means of detection, demonstration by culture being next best, and the classical Z-N staining technique to be least sensitive.

Bachmann & *Finke* (*1939*) were the first to apply fluorescent staining to tissue sections, and were thereby able to demonstrate greater numbers of tubercle bacilli in tissue than otherwise possible in Z-N stained sections They also pointed out the superiority of the fluorescence method for the detection of scattered bacteria in tissue sections *Gray* (1953) stated that the fluorescence method *reveals even five times the number of orga* nisms otherwise revealed by the Z-N technique in adjacent sections from a block of tissues Other comparisons between staining methods applied to tissue sections and culture (*Kuper* & *May* 1960, *Braunstein* & *Adriano* 1961, *Truant et al* 1962, *Koch* & *Cote 1965*, *Yamaguchi* & *Braunstein 1965*, *Richter* 1969), mainly carried out on human material, have yielded similar results

If, however, the fluorescent technique is applied to such specimens its specifity may be doubted *Wen Lan Lou Wang* (1969) reported that the appearance of acid fast bacilli in many of the sections could be due to contamination of the paraffin by non specific acid-fast bacilli He suggested that these contaminants may multiply in the paraffin and be present in the paraffin blocks from which tissue sections are made

We have tried to maintain control over specificity, using a single well defined strain, H_3 Rv, in our experimental studies in guinea pigs The risk of confusion with atypical acid-fast bacilli would seem to be virtually nil The work was conducted under strictly aseptic conditions, using newly sterilized instruments for each organ at operation, autopsy and in preparation of material for cul ture New paraffin was always used for embedding A paraffin dispenser or Autotechni *con was not used*

Under these experimental conditions we have been able to confirm the superiority of the fluorescence method over the classical Ziehl-Neelsen method for the detection of tubercle bacilli Not only the method has a higher accuracy but the staining technique is simpler and the microscopy more rapid Above all, the fluorescent technique was va*luable if applied to organs in which only a* few bacteria were present, this has previously been pointed out by *Bachmann* & *Finke* (1939), *Haebler* & *Murray* (1954) and by *Parrot et al* (1970) The great reduction in the time required for examination, compared with that required for Z-N stained sections, was a major advantage. *Larsen* (*1940*) estimated, using fluorescent technique, that it was possible *to examine* a 10 times larger area in less than 1/3 the time required for exa mination of Z N stained specimens The brilliance of the bacteria makes it possible to identify them at a lower magnification, the use of oil immersion is not necessary

The high frequency of negative Z N stain*ing in spite of positive fluorescence micro*scopy (37 specimens) is probably explained in part by the fact that we limited our inspection to 30 minutes A thorough examina tion of Z-N stained specimens under oil im mersion is time-consuming, requiring up to 8 hours per specimen, according to some au thors (*Bogen 1941*)

The most striking features of bacteria examined by the fluorescence technique are their brilliance and the clarity with which their morphology is demonstrated which make it possible to identify them at a lower magnification, the use of oil immersion is not necessary These qualities make *it easy to* distinguish the bacteria from artefacts The fluorescence of artefacts is usually less and

Acta path. microbiol. scand Section A. 81, 831-842, 1973

THE ADRENAL GLANDS OF ELDERLY MEN IN RELATION TO ABNORMAL PROSTATIC GROWTH

An Analysis in an Autopsy Series

OLAV A. HAUGEN

Ullevål Hospital, Department of Pathology, University of Oslo, Oslo, Norway
Head Kristen Arnesen, M.D.

The variability of adrenal weight and cortical thickness was studied in a consecutive autopsy series comprising 154 men over 40 years of age. Among several factors tested by multiple regression analysis, a significant and positive correlation between adrenal weight and body length was demonstrated only. At all age levels and within all groups of prostatic histology the adrenal weights varied markedly. No significant association between the adrenal parameters studied, including the presence of adreno-cortical adenomas, and any of the defined groups of prostatic histology was found in this series. With advancing age, however, a significant decrease of adrenal weight appeared in men with prostatic carcinoma which was not seen in men with benign hyperplasia. The validity and importance of this observation is obscure. It is concluded on the basis of morphological studies of the adrenal glands that there is no firm indication to suggest that the adrenal glands are involved in the pathogenesis of abnormal growth of the prostate in elderly men.

The extent to which the pituitary adrenal axis is involved in the development of benign hyperplasia and neoplasia of the prostate in man is uncertain. Although some patients with advanced stages of prostatic carcinoma may benefit from hypophysectomy or adrenalectomy (*Huggins & Scott* 1945, *Reynoso & Murphy* 1972), no conclusive evidence has yet been presented which suggests that pituitary or adrenal hormones play a decisive role in the pathogenesis of age associated abnormal prostatic growth.

Adrenocorticotrophic hormone (ACTH) is responsible for the maintenance of cortical structure and function, long term stimulation causing hyperplasia with excessive production of both glucocorticoid hormones and androgenic substances (*Liddle* 1971, *Migeon* 1972). A growth promoting effect of ACTH upon the reproductive organs of rats has been observed by some investigators, while others have been unable to show any such effect (*Tullner* 1963). Experimental studies in mice have suggested that hypercorticoidism may be involved in oestrogen induced benign prostatic hypertrophy (*Fingerhut & Veenema* 1966, 1967), and cortisone has been shown to enhance the growth stimulating effect of oestradiol on the prostate in rats (*Tisell* 1971). However, in view of the great physiological differences between rodents and man, these results cannot be extrapolated to the human situation.

The weight and morphology of the adrenal

Received 25.vi.73 Accepted 25.vi.73

Requests for reprints should be addressed to: Department of Pathology, Ullevål Hospital, Oslo 1, Norway.

glands studied in autopsy material apparently are in favour of increased cortical activity in men with abnormal growth of the prostate (*Mellgren* 1945, *Chwalla* 1954, *Sommers* 1957, *Koppel et al* 1967) Limited data from patients adrenalectomized for prostatic carcinoma, however, have suggested that the adrenal glands of these patients are entirely normal (*Huggins et al* 1953)

The present investigation is part of a more extensive study of the relationship between pathological growth of the prostate and the weight and morphological characteristics of the pituitary gland, the adrenal glands and the testes In previous reports, the pituitary weight (*Haugen* 1973a), the distribution of pituitary cell types (*Haugen* 1973b) and the frequency of pituitary adenomas (*Haugen* 1973c) in this series have been described From these studies it appeared that some form of pituitary hyperactivity could be a characteristic of men with hyperplasia or neoplasia of the prostate The histological findings in the prostate in this series have also been previously described (*Harbitz* & *Haugen* 1972)

MATERIAL AND METHODS

The adrenal glands and the prostates from 207 consecutive autopsies of men over 40 years of age were collected during a 3 month period 1967 68 In the main analysis of the adrenal weight 53 patients were excluded for the following reasons previous prostatic surgery (24 patients) oestrogen treatment for clinically manifest prostatic carcinoma (6 patients) secondary tumour invasion of the prostate or testes (4 patients) seminoma of the testis (one patient) secondary tumour deposits in the adrenal glands (11 patients) adrenal haemorrhages (3 patients) and secondary amyloidosis of the adrenal glands (one patient) In three instances the adrenal glands were severely damaged at autopsy thus leaving a total of 154 pairs of glands to be included in the analysis of adrenal weight In addition 10 pairs of glands were regarded as unsuitable for measurement of cortical width

The adrenal glands were dissected free from fat tissue as thoroughly as possible and the *combined weight* of the unfixed glands was recorded The glands were fixed in 10 per cent neutral formaldehyde solution for 10 days after which the glands were cut in thin slices The presence of cortical adenomas (single or multiple nodules exceeding 5 mm) was recorded, and from each gland a transverse block (3-4 mm thick) through the central part of the adrenal body was taken for measurement of cortical width If in cases of adenomas, these were not included in the standard section additional sections were embedded and the presence of cortical elements in the adenomas microscopically confirmed Gland pairs showing secondary tumour deposits in any part were rejected Histological examination and measurement of cortical width were performed on sections stained with haematoxylin and eosin All sections carried identification numbers and marks for right' and left respectively

Measurement of Cortical Width

The cortical width including all zones from the capsule to the adrenal medulla, was measured with a microscope equipped with an ocular micrometer In each gland seven randomly selected points equally distributed round the circumference were measured Thus the mean cortical width of one pair of glands was based on values recorded at 14 different points Sections including cortical nodules (adenomas) were also measured in a similar way Examination of the adrenal glands was performed without any knowledge of the clinical data

Prostate

The procedures of dissection and histological examination of 3 6 total transverse sections from each prostate have previously been described (*Harbitz* & *Haugen* 1972) The presence of benign nodular hyperplasia (BNH) carcinoma (C) atypical glandular proliferation (AGP) or diffuse atrophy (DA) was noted for each prostate The presence of AGP in prostates showing carcinoma was not recorded A histologically normal prostate (N) showed none of the characteristics mentioned above

The histological findings in the prostates of 154 patients included in the main analysis appear from Table 1

Clinical Data

Clinical data were recorded from the clinical notes and prepared for the computer analysis Apart from eight patients with clinically manifest diabetes mellitus none were known to suffer from other endocrine disorders

Statistical Methods

Modified Student s t tests accounting for unequal variances and numbers of individuals were used for testing differences between arithmetic means and for testing differences between slopes

TABLE 1 *Histological Diagnoses of the Prostate in 145 Patients**

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	1	0	2	0	0	0	0
50–59	9	4	10 (9)	3 (2)	1	4	0
60–69	6	1	22 (21)	13 (12)	4	6	1
70–79	0	1	25	17 (15)	0	4 (3)	1
80 +	0	0	8	9 (6)	0	2	0
	16	6	67 (65)	42 (35)	5	16 (15)	2

* N – normal histology, DA – diffuse atrophy, BNH – benign nodular hyperplasia, C = carcinoma, AGP – atypical glandular proliferation

Numbers in brackets indicate number of cases in which measurement of adrenal cortical width was performed

of regression lines (*Snedecor & Cochran* 1967) n_A and n_B being the number of observations in the groups to be compared, p values were based on the least of n_A 1 and n_B 1 (for means) and n_A 2 and n_B 2 (for slopes) degrees of freedom *P values* below 0 05 were regarded statistically significant

Multiple linear regression analysis Stepwise and full multiple regression analysis were applied as previously described (*Haugen & Harbitz* 1972, *Haugen* 1973a) using the adrenal weight and the adrenal cortical width respectively as *dependent variables* (X_1) The following factors either bivariate (1 0, labelled X_2 to X_{12}) or continuous (labelled X_{13} to X_{15}) were treated as *explanatory* (independent) variables

Histology of the Prostate

- X_2 Benign nodular hyperplasia (BNH)
- X_3 Atypical glandular proliferation (AGP)
- X_4 Carcinoma (C)
- X_5 Diffuse atrophy (DA)

Cause of Death

- X_6 Cardiovascular disease*
- X_7 Malignant tumour

Duration of Final Illness

- X_8 1 7 days
- X_9 >7 days

* Includes death from myocardial infarction (47 cases) cerebrovascular and peripheral vascular disease (16 + 8 cases) rheumatic valvular disease (4 cases) miscellaneous cardiovascular disorders (11 cases)

Other

- X_{10} Steroid hormone treatment§
- X_{11} Diabetes mellitus
- X_{12} Liver cirrhosis
- X_{13} Age
- X_{14} Body weight
- X_{15} Body length

Initially, forward stepwise regression analysis was run until all explanatory variables which were partially significant at the 5 per cent level at each step, were included Thereafter, the selected variables together with all groups of prostatic histology (X_2 to X_5) were included in the full multiple regression analysis Differences between the regression coefficients for the various groups of prostatic histology were tested by an F test (*Scheffé* 1959)

The analysis was based on a standard program for multiple regression analysis (NRSR) developed at The Norwegian Computing Center, Oslo, and was conducted on a Univac 1108 computer

Reproducibility The reproducibility of the method applied for *measurement of cortical width* was tested by duplicate measurements of 10 pairs of randomly selected adrenal glands These sections were drawn by another member of the staff and mixed with sections which had not been measured previously During all measurements, the identification number of the sections were covered with tape and there was a time lag of several weeks between first and second measurement The results have been plotted in Fig 1 The *method error* was computed from the usual formula $\sqrt{\frac{\Sigma D^2}{2n}}$,

§ Includes treatment with corticosteroids (prednisone) (5 cases) anabolic steroids (nortestosterone) (5 cases) or both (8 cases)

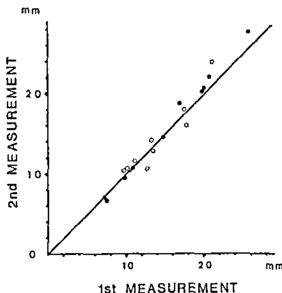


Fig 1 Scatter plot showing duplicate measurements of adrenal cortical width, symbols refer to *the right (●) and left (O) adrenal gland respectively*

where D denotes the difference between duplicates and n the number of pairs compared The method error calculated separately for measurements of the right and left adrenal was 0 04 and 0 07, respectively This indicates that inaccuracies in measurements of cortical width probably was a negligible source of error

RESULTS

The adrenal weight showed an approximately *normal distribution (Fig 2A) while the distribution* of the cortical width was slightly skew (Fig 2B)

As appears from Table 2, *the mean* adrenal weight showed a slight decrease with age, but the negative relationship to age was not statistically significant (Table 6) The cortical width was apparently unrelated to age (Tables 2 and 6)

Table 3 presents adrenal weight and cortical width in relation to cause of death, duration of final illness steroid hormone treatment, diabetes mellitus and liver cirrhosis *The mean adrenal weights among patients* dying from cardiovascular disease or malignant tumours were practically the same, both being lower but not significantly different from that observed in patients dying from other causes ($p>0.05$ and $p>0.10$, respectively) The mean cortical width among patients dying from malignant tumours was significantly lower than that observed in patients dying from other conditions ($p<0.02$) Ten patients dying from malignant tumours had received corticosteroid hormones (Prednisone) The cortical width in these *patients (1 24±0 30 mm) was only sligthly* lower than that observed in cancer patients not treated with such agents (1 27±0 28 mm) In the group of cardiovascular deaths, the mean adrenal weight among 19 patients

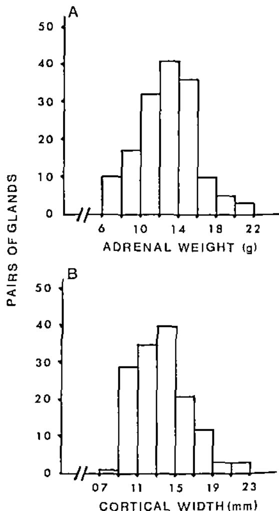


Fig 2 Distribution of adrenal weight (g) among 154 individuals (A) and adrenocortical width (mm) among 144 individuals (B)

TABLE 2 *Adrenal Weight (g) and Cortical Width (mm) in Relation to Age*

Age	Adrenal weight			Cortical width		
	No of patients	Mean	S D	No of patients	Mean	S D
40–49	3	11 80	3 38	3	1 31	0 25
50–59	31	13 36	2 54	29	1 38	0 32
60–69	53	12 95	2 96	51	1 33	0 28
70–79	48	12 55	3 09	45	1 33	0 28
80+	19	12 21	3 67	16	1 36	0 25
Total	154	12 84	3 01	144	1 34	0 28

S D Standard deviation

TABLE 3 *Weight (g) and Cortical Width (mm) of the Adrenal Glands in Relation to Cause of Death, Duration of Final Illness, Steroid Hormone Treatment, Diabetes Mellitus and Liver Cirrhosis*

	No of patients	Adrenal Weight		No of patients	Cortical Width	
		Mean	S D		Mean	S D
Cause of death						
Cardiovascular disease	86	12 55	2 81	79	1 33	0 25
Malignant tumour	32	12 51	3 59	30	1 26	0 28
Other conditions	36	13 63	2 87	35	1 44	0 31
Duration of final illness						
<1 day	37	12 17	2 74	35	1 34	0 26
1 7 days	32	13 17	2 73	30	1 39	0 30
>7 days	85	12 93	3 21	79	1 33	0 28
Steroid hormone treatment	18	12 21	3 63	16	1 27	0 32
Diabetes mellitus	8	14 80	3 01	7	1 41	0 22
Liver cirrhosis	3	12 90	4 42	3	1 68	0 44

S D Standard deviation

with cardiac hypertrophy (heart weight exceeding 500 g) and established hypertension

mal heart weight The difference between means was not statistically significant (p> 0 20) Only a few patients had received long-term and high-dose treatment with corticosteroids which probably explains the great variability of cortical width (range 0 91-1 79 mm) observed in this category of patients After short-term illness both the weight and cortical thickness of the adrenal glands appeared to be higher than that seen in cases of "sudden deaths" The differences between means, however, were not statistically significant (p>0 10 and p>0 40 respectively) The few diabetics showed high adrenal weight and increased cortical width compared to the values observed in the total series

The mean adrenal weight and cortical width in relation to the histology of the prostate are presented in Table 4 The low adrenal weight observed among patients with BNH was not significantly different from that of patients with a histologically normal prostate (p>0 10) The highest adrenal weight

TABLE 4 *Adrenal Weight (g) and Cortical Width (mm) in Relation to Histology of the Prostate**

Histology of the prostate	No of patients	Adrenal weight Mean	Adrenal weight SD	No of patients	Cortical width Mean	Cortical width SD
N	16	13 49	2 43	16	1 41	0 32
DA	6	13 33	2 84	6	1 24	0 16
BNH	67	12 45	2 89	65	1 34	0 29
C+BNH	42	13 04	3 50	35	1 37	0 25
C	5	15 44	2 98	5	1 58	0 44
AGP+BNH	16	12 86	2 17	15	1 24	0 18
AGP	2	8 15	0 91	2	0 97	0 28
	154	12 84	3 01	144	1 35	0 28

* For abbreviations, see Table 1
SD Standard deviation

was encountered in patients carrying C alone, but their adrenal weight did not differ significantly from that among patients with C+BNH (d f =4, $p>0\ 10$) or BNH (d f = 4, $p>0\ 05$) The cortical width in patients with BNH and C+BNH was approximately the same, while in relation to DA and AGP the cortical width was slightly lower

Within all histological groups and at all age levels a great variation of the adrenal weights was apparent (Fig 3) The most extreme variation occurred in relation to prostatic carcinoma (range 6 48 21 7 g) The regression lines for adrenal weight on age within the main groups of prostatic histology are presented in Fig 4 The regression coefficient for C+BNH alone ($b = -0\ 121$) was significantly different from zero ($p<0\ 05$), and also differed significantly from that in men with BNH ($b=0\ 042$) ($t=2\ 424$, d f =40, $p<0\ 02$)

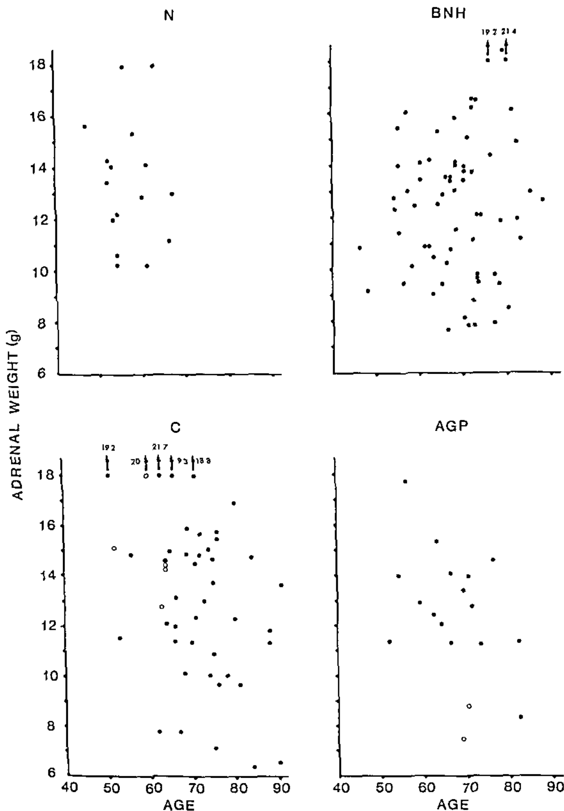
The frequencies of adrenal cortical adenomas in relation to the histology of the prostate appear from Table 5 Obviously, cortical adenomas were not more common in patients with prostatic carcinoma (C and C+BNH) than among patients with BNH Furthermore, the frequencies of cortical adenomas in patients with normal prostate and BNH did not differ significantly from each other ($X^2=0\ 17$, $p>0\ 60$)

Multiple Regression Analysis

Simple correlation analysis was performed as part of the regression analysis (Table 6) A significant and positive correlation between adrenal weight (X_1) and body length (X_{16}) was observed only A negative and statistically significant correlation between the cortical width and the presence of AGP (X_3) was observed, while there was a positive correlation to liver cirrhosis (X_{12})

The results of the full regression analysis, including all groups of prostatic histology and factors selected at the stepwise procedure, are presented in Table 7 None of the histological groups showed any significant correlation to adrenal weight or the cortical width By including body length and the histological diagnoses in the regression analysis, only about 6 per cent of the variation in adrenal weight could be explained ($R^2=0\ 06$), the explanatory value of these factors in the analysis of cortical width variability being slightly above 7 per cent ($R^2=0\ 075$)

Fig 3 Scatter plots showing adrenal weights (g) in relation to age of men with histologically normal prostate (N), benign nodular hyperplasia alone (BNH), carcinoma (C) and atypical glandular proliferation (AGP) of the prostate *O* refers to subjects in which BNH was not present


N
BNH
C
AGP
ADRENAL WEIGHT (g)
AGE
AGE

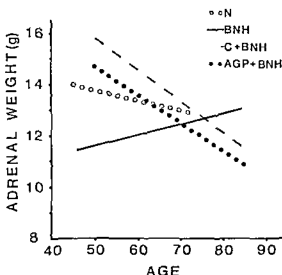


Fig 4 Regression lines for adrenal weight on age in different groups of prostatic histology Normal histology (N), benign nodular hyperplasia alone (BNH), carcinoma with hyperplasia (C+BNH) and atypical glandular proliferation with BNH (AGP+BNH)

COMMENT

It is well known that the weight of the adrenal glands recorded post mortem *(Roessle & Roulet* 1932, *Sommers* 1957) by far exceeds that of adrenal glands removed surgically from patients without endocrinopathies (*Studzinsky et al* 1963) The weight of a single "normal" adrenal gland at autopsy has been assessed to be 6 6 5 grams (*Symington* 1969) which is approximately 30 per cent more than the weights found in cases of sudden accidental deaths (*Quinan & Berger* 1933 *Bloodworth* 1966) Although the mean value of adrenal weight observed in the present series apparently is within the range of "normality" for autopsy populations, a large proportion of the observations falls outside this range It was assumed that a number of factors could be partly involved in the variablity observed and multiple regression analysis was performed in an attempt to account for the influence of such factors

The influence of stress of varying intensity may be the main reason for the increase of adrenal mass *(Symington* 1969) ACTH-stimulation during 3 4 days may increase the adrenal weight by more than 100 per cent and induce morphological changes similar to those seen after severe and prolonged stress (*Studzinsky et al* 1962) Although statistically unproven, the increase of weight and cortical width observed in patients included in the present series who died after short-term illness are in consistency with the concept of stress-induced adrenocortical hyper plasia The reasons for the unexpectedly high adrenal weights in cases of "sudden deaths" are not obvious The majority of patients in this group died from coronary heart disease and it is conceivable that some may have suffered from hypertension which may be associated with adrenocortical hyperplasia (*Rusell & Masi* 1970)

Adrenocortical hyperplasia has also been reported to be particularly frequent in patients with malignant tumours (*Parker & Sommers* 1956, *Shaliton et al* 1961) Al though the number of patients dying from malignant tumours was small in the present series, there was no indication of altered weight, but the cortical width was even lower in cancer patients compared with non cancer controls

According to *Christian* (1953) there is a log linear relationship between adrenal weight and body weight in most species, including man This relationship could not be demonstrated in the present series (*Haugen*, unpub-

TABLE 5 *Recorded Frequency of Adrenal Cortical Adenomas in Relation to the Histology of the Prostate**

Histological diagnoses	No of patients	No of patients with adenomas	Per cent
N	16	1	6 3
DA	6	1	16 7
BNH	67	10	14 9
C+BNH	42	5	11 9
C	5	1	20 0
AGP+BNH	16	1	6 3
AGP	2	0	–
All	154	19	12 3

* For abbreviations, see Table 1

TABLE 6. *Relationship between Adrenal Weight and Cortical Width and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variables	Dependent variables (X_1) Adrenal weight (n_1 = 154)	Cortical width (n_1 = 144)
	Correlation coefficients	
*Histology of the prostate**		
X_2 BNH (n = 125, 115)	−0 093	−0 049
X_3 AGP (n = 16, 15)	−0 056	−0 177†
X_4 C (n = 47, 40)	0 098	0 112
X_5 DA (n = 6, 6)	0 033	−0 080
Cause of death		
X_6 Cardiovascular disease (n = 86, 79)	−0 091	−0 039
X_7 Malignant tumour (n = 32, 30)	−0 052	−0 154
Duration of final illness		
X_8 1 7 days (n = 32, 30)	0 066	0 096
X_9 >7 days (n = 85, 79)	0 049	−0 074
Other		
X_{10} Steroid hormone treatment (n = 18, 16)	−0 072	−0 089
X_{11} Diabetes mellitus (n = 8, 7)	0 157	0 050
X_{12} Liver cirrhosis (n = 3, 3)	−0 006	0 173†
X_{13} Age (n = 154, 144)	−0 123	−0 054
X_{14} Body weight (n = 154, 144)	0 039	−0 029
X_{15} Body length (n = 154, 144)	0 220†	0 053

* For abbreviations, see Table 1

n_1 Number of cases in which adrenal weight or cortical width were recorded

n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continuous variables) The first figure refers to number of cases included in the adrenal weight analysis, the second figure refers to cortical width analysis

† Significant at the 5 per cent level

lished data) whereas there was a positive and significant relationship to body length It thus appears as if body length variations should be accounted for in the analysis of adrenal weight variability at autopsy

From the age of 50 years there was a slight reduction in the mean adrenal weight Although the negative association to age was not statistically significant, the data obtained in the present series accord with similar observations by *Roessle & Roulet* (1932) Functional studies (*West et al* 1961) have indicated that the adrenal cortex becomes less responsive to ACTH stimulation with ageing There is also a marked decline in the urinary excretion of 17 hydroxysteroids and 17-keto steroids with advancing age in both sexes (*Romanoff et al* 1957, *West et al* 1961) but no decrease of plasma cortisol (*Peterson* 1971) Among the androgens produced by the adrenals, all of which are biologically weak androgens (*Migeon* 1972), dehydro-epiandrosterone also decreases with age (*Gandy & Peterson* 1968) Whether the decreased adrenal weight seen in high age actually is an expression of altered reponsiveness or decreased functional capacity cannot be answered on the basis of this analysis

The main purpose of the present investigation was to analyse the adrenal parameters in relation to various defined groups of prostatic histology Contrasting previous studies (*Mellgren* 1945, *Chwalla* 1954, *Sommers* 1957, *Koppel et al* 1967), the present findings do

TABLE 7 *Adrenal Weight and Cortical Width in Relation to the Histology of the Prostate Full Regression Analysis§*

a Explanatory variables	Adrenal weight (X_1, $n_1 = 154$)		
	Partial correlation coefficient	Partial regression coefficient	Significant at level
X_{15} Body length (n=154)	0 197	0 091	0 016
X_4 C (n-47)	0 066	0 445	0 424
X_2 BNH (n=125)	-0 058	-0 491	0 479
X_3 AGP (n=16)	-0 031	-0 298	0 701
X_5 DA (n=6)	0 023	0 387	0 779
Multiple correlation coefficient (R)	0 245		

b Explanatory variables	Cortical width (X_1, $n_1 - 144$)		
	Partial correlation coefficient	Partial regression coefficient	Significant at level
X_{12} Liver cirrhosis (n-3)	0 159	0 311	0 060
X_3 AGP (n=15)	-0 154	0 136	0 070
X_5 DA (n=6)	-0 106	-0 161	0 215
X_2 BNH (n-115)	-0 079	-0 060	0 352
X_4 C (n-40)	0 068	0 044	0 421
Multiple correlation coefficient (R)	0 275		

§ For abbreviations of histological diagnoses see Table 1

not suggest that the adrenal glands of men with abnormal prostatic growth are markedly different from those of men with a histologically normal prostate With advancing age, however, it appeared that the adrenal weight showed a marked decrease in men with carcinoma or atypical glandular proliferation accompanied by BNH The significance and validity of these observations are uncertain, but they may be due to the small number of patients with AGP and the extreme variation of adrenal weight in patients with prostatic carcinoma

Fingerhut & *Veenema* (1966) reported that oestrogen administration to male Strong A/J mice induced benign prostatic enlargement with nodular fibrous tissue and glandular proliferation All of these animals showed marked adrenal enlagement (*Fingerhut* & *Veenema* 1967) with hyperplasia of the zona glomerulosa These authors suggested that increased function of the adrenal glands, set up via the hypophysis as a result of atrophy of the testicular germinal tissue, could explain the development of benign hyperplasia In men comprised in the same series as that reported in the present study, it was previously observed that the proportion of PAS positive cells of the adenohypophysis some of which produce ACTH, and the frequency of small pituitary adenomas was higher in men with carcinoma or hyperplasia of the prostate than among normal controls (*Haugen* 1973b, 1973c) Since the adrenal weight and cortical width were not markedly different in these conditions, it is unlikely that the morphological findings in the adenohypophysis can be related to altered adrenal states in these patients

Most adrenal androgens have limited biological activity, and in normal men, androstenedione contributes only by about 15 per

cent to the production rate of dihydrotestosterone which probably is the biologically active form of androgens in men (*Migeon* 1972) On the basis of the study of the prostates from patients with Addison's disease, *Moore* (1947) concluded that the adrenal gland activity in adults did not affect the structure of the prostate Limited data available from a study of patients with Klinefelter's syndrome (*Nielsen* &*Perboll* 1969) showed the prostates to be normal in high age, the adrenal glands of these patients being entirely normal while their testes showed marked atrophy

In accordance with *Huggins et al* (1953) it may be concluded that the present study failed to provide any firm indications by which to support the view that the adrenal glands of men with abnormal prostatic growth are different from the adrenal glands of men with a histologically normal prostate

---

Actuary *Ingar Holme*, Cand real, The Norwegian Computing Center, Oslo, Norway performed the computer analysis and gave valuable advice. The technical assistance of Mrs *Inger Johanne Ingebrigtsen* is gratefully acknowledged

The investigation was supported by grants from *Professor Carl Sembs Fond* and *Statssekretær Jacob Aall og hustrus legat*

REFERENCES

1 *Bloodworth, J M B* The adrenal In Sommers S C (Ed) Pathology Annual Appleton Century Crofts Inc, New York, 1966 p 172–192

2 *Christian, J J* The relation of adrenal weight to body weight in mammals Science *117* 78 80, 1953

3 *Chwalla, R* Untersuchungen uber die endokrine Abhangigkeit der diffusen Prostatahyperplasie und des Prostataadenoms Verh bericht der Deutschen Gesellsch. fur Urologie Z Urol (Sonderheft) 1954 p 339–385

4 *Fingerhut, B & Veenema, R J* Histology and radioautography of induced benign enlargement of the mouse prostate Invest Urol *4* 112–124 1966

5 *Fingerhut, B* & *Veenema, R J* The effect of bilateral adrenalectomy on induced benign prostatic hyperplasia in mice J Urol *97* 508–517, 1967

6 *Gandy H M* & *Peterson, R E* Measurement of testosterone and 17 ketosteroids in plasma by the double isotope dilution derivate technique J clin Endocr *28* 949–977, 1968

7 *Harbitz, T B* & *Haugen, O A* Histology of the prostate in elderly men A study in an autopsy series Acta path microbiol scand Section A *80* 756 768, 1972

8 *Haugen, O A* & *Harbit, T B* Prostatic weight in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *80* 769–777, 1972

9 *Haugen, O A* Pituitary weight and the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *81* 204–212, 1973a.

10 *Haugen, O A* Distribution of pituitary cell types in relation to the histology of the prostate in elderly men An analysis in an autopsy series Acta path. microbiol scand Section A *81* 411–424, 1973b

11 *Haugen, O A* Pituitary adenomas and the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *81* 425–434, 1973c

12 *Haugen, O A* Unpublished data 1973

13 *Huggins, C* & *Scott, W W* Bilateral adrenalectomy in prostatic cancer Clinical features and urinary excretion of 17 ketosteroids and estrogen Ann Surg *122* 1031–1041, 1945

14 *Huggins, C C, Bergenstal, D M* & *Cleveland Anne S* The adrenal in cancer Recent Progr Hormone Res *8* 273–282, 1953

15 *Koppel, M, Meranzie, D R* & *Shimkin, M B* Characteristics of patients with prostatic carcinoma a control case study on 83 autopsy pairs J Urol *98* 229 233, 1967

16 *Liddle, G W* Regulation of adrenocortical function in man In Christy, N P (Ed) The human adrenal cortex Harper & Row, Publishers New York, Evanston San Francisco London 1971 p 41–68

17 *Mellgren, J* The anterior pituitary in hyperfunction of the adrenal cortex. An anatomical study with special reference to syndroma Morgagni and notes on prostatic hypertrophy Acta path microbiol scand (Suppl) *60* 1–177 1945

18 *Migeon, C J* Adrenal androgens in man Amer J Med *53* 606–626, 1972

19 *Moore, R A* Benign hypertrophy and carcinoma of the prostate In Twombly, G H & Pack G T (Eds) Endocrinology of neoplastic diseases A symposium by eighteen authors Oxford University Press New York 1947 p 194 212

20 *Nielsen, J* & *Perboll, O* Autopsy of patients with Klinefelter's syndrome Dan med Bull *16* 10–15 1969

21 *Parker, T G* & *Sommers, S C* Adrenal

cortical hyperplasia accompanying cancer Arch Surg *72* 495–499, 1956

22 *Peterson, R E* Metabolism of adrenal cortical steroids In Christy, N P (Ed) The human adrenal cortex Harper & Row, Publishers. New York, Evanston, San Fransisco, London, 1971 p 87–189

23 *Quinan, C & Berger, A A* Observations on human adrenals with especial reference to relative weight of normal medulla Ann intern Med *6* 1180–1192, 1933

24 *Reynoso, G & Murphy, G P*, Adrenalectomy and hypophysectomy in advanced prostatic carcinoma Cancer *29* 941–945, 1972

25 *Roessle, R & Roulet, F* Mass und Zahl in der Pathologie Springer, Berlin und Wien, 1932 p 76–79

26 *Romanoff, L P Wolf, R & Pincus, G* A comparison of urinary steroid excretion patterns in young and elderly subjects before and after ACTH administration J Geront *12* 394–397, 1957

27 *Russel, R P & Masi, A T* The prevalence of adrenal cortical hyperplasia at autopsy and its association with hypertension Ann intern Med *73* 195–205, 1970

28 *Shelton, L J Ince, J S & Werk, E E Jr* Adreno cortical width in carcinoma of the lung Cancer *14* 105–110, 1961

29 *Scheffe H* The analysis of variance John Wiley & Sons, Inc, New York 1959 p 25–51

30 *Snedecor, G W & Cochran, W G* Statistical methods 6th ed The Iowa State University Press, Ames Iowa 1967, p 114–116

31 *Sommers, S C* Endocrine changes with prostatic carcinoma Cancer *10* 345–358, 1957

32 *Studzinski, G P, Symington, T & Grant, J K* Triphosphopyridine nucleotide linked dehydrogenases in the adrenal cortex in man, the effect of corticotrophin and the distribution of enzymes Acta Endocr (Kbh) *40* 232–246, 1962

33 *Studzinski, G P, Hay, D C F & Symington, T* Observations on the weight of the human adrenal gland and the effect of preparations of corticotropin of different purity on the weight and morphology of the human adrenal gland J clin Endocr 248 254, 1963

34 *Symington, T* The adrenal cortex In Symington, T Functional pathology of the human adrenal gland E & S Livingstone Ltd, Edinburgh and London, 1969 p 3–216

35 *Tisell, L E* The growth of the ventral prostate the dorsolateral prostate, the coagulating glands and the seminal vesicles in castrated adrenalectomized rats injected with oestradiol and/or cortisone Acta Endocr (Kbh) *68* 485–501, 1971

36 *Tullner, W W* Hormonal factors in the adrenal dependent growth of the rat ventral prostate In Vollmer, E P & Kauffmann, Gladys (Eds) Biology of the prostate and related tissues Nat Cancer Inst Monogr *12* 211–224 1963

37 *West C D Brown, H Simons E L Carter, D B Kumagai, L F & Englert E* Adrenocortical function and cortisol metabolism in old age J clin Endocr *21* 1197–1207, 1961

Acta path microbiol scand Section A 81, 843-856, 1973

MORPHOMETRIC STUDIES OF THE SEMINIFEROUS TUBULES IN ELDERLY MEN WITH SPECIAL REFERENCE TO THE HISTOLOGY OF THE PROSTATE

An Analysis in an Autopsy Series

THORSTEIN B HARBITZ

Ullevål Hospital Department of Pathology, University of Oslo, Oslo, Norway
Head Kristen Arnesen, M D

The weight of the seminiferous tubules (W_{Tu}) was estimated and analysed in a consecutive autopsy series of men over 40 years This series also formed the basis for a comprehensive study of morphological characteristics of the testes the pituitary gland and the adrenal glands in men with prostatic hyperplasia and neoplasia Among 172 patients who had not previously undergone prostatic surgery or received oestrogenic hormones, W_{Tu} varied considerably at all ages and showed no significant alteration with advancing age At simple and multiple regression analysis, body weight and death from cardiovascular disease were positively correlated with W_{Tu}, whereas protracted terminal illness apparently reduced W_{Tu} significantly W_{Tu} *per se* showed no relation to the presence of benign nodular hyperplasia (BNH) or carcinoma (C) of the prostate, and did not discriminate between the various forms of abnormal prostatic growth encountered However patients with BNH generally showed a conspicuous reduction in W_{Tu} with age whereas C was associated, to a larger extent, with preserved tubular mass in advanced years W_{Tu} was moderately lowered in 24 men who had been subjected to prostatic surgery, and oestrogen treatment for prostatic carcinoma apparently resulted in severe degeneration of the seminiferous tubules The results are in accordance with the assumption that some form of endocrine impairment at the gonadal and/or pituitary level is involved in the pathogenesis of prostatic hyperplasia and neoplasia

The pathogenesis of benign nodular hyperplasia and carcinoma of the prostate is as yet not clear There are reasons to believe, however, that age related changes in the functions of the pituitary gland, the adrenal glands and the testes are of importance

Observations in a previous autopsy study (*Harbitz* 1973 a) suggested a possible relationship between the histology of the prostate, testis weight and age A major portion of the testicular volume is constituted by the seminiferous tubules (*Roosen-Runge* 1956), and testicular size has been suggested as a rough measure of spermatogenesis and tubular function (*Prader* 1966) The maintenance of spermatogenesis normally depends upon adequate stimulation from androgenic hor-

Received 10 vii 73 Accepted 10 vii 73

Requests for reprints should be addressed to *Department of Pathology, Ullevål Hospital Oslo 1*

mones (*Walsh et al* 1934, *Nelson & Merckel* 1937, *Boccabella* 1963), and the seminiferous tubules apparently represent a sensitive indicator of disturbances in the normal balance between androgens and oestrogens both in experimental animals and man (*Heller et al* 1950, *Ludwig* 1950, *de la Balze et al* 1954, *Sniffen et al* 1954, *Bacon & Kirkman* 1955) Hence, an investigation of the relations between the amount of tubular tissue and the histology of the prostate might give additional information concerning the pathogenesis of abnormal growth of the prostate in elderly men

This investigation forms part of a comprehensive autopsy study of the relations between pathological growth of the prostate and certain morphological characteristics in the testes, the pituitary gland and the adrenal glands in elderly men Single variable and multiple regression analyses have been used in order to investigate and correct for factors (confounding variables) which may interfere with the morphology of endocrine glands and disturb the usefulness of the seminiferous tubules as a measure of endocrine function

MATERIALS AND METHODS

The testes and prostates for the present study were collected at the consecutive autopsies of 207 men over 40 years of age who died at Ullevål Hospital during a 3 months period 1967–68 For details, see previous reports on the same series of patients by *Harbitz & Haugen* (1972) and *Harbitz* (1973 a, b) Again, five patients were unsuited for further analysis and were excluded, and patients who had undergone prostatic surgery (24), treatment with oestrogenic hormone (diethylstilboestrol) (4) or both (2) were analysed separately Thus, the main analysis of spermatogenic tubules was based upon 172 cases

Prostate

The presence of normal histology (N), benign nodular hyperplasia (BNH), carcinoma (C), atypical glandular proliferation (AGP) or diffuse atrophy (DA) of the prostate was noted for each gland The occurrence of atypical glandular proliferation was not specified in glands where carcinoma was diagnosed The histological findings in the prostate of the 172 cases included in the main analysis are presented in Table 1

Seminiferous Tubules

Three total sections from different levels of the testes were stained with phosphotungstic acid haematoxylin (PTAH) and examined under the light microscope at 10x objective magnification The proportion of the testicular tissue constituted by seminiferous tubules was assessed by point sampling in a restricted number of visual fields as previously described (*Harbitz* 1973 b)

A total number of 972 intersection points was generally (for exceptions, see *Harbitz* 1973 b) counted in each testis All points projecting inside the tubular basement membrane, whether falling on germinative epithelium or tubular lumen, were counted as 'hits Points falling on completely sclerosed tubules were not counted The number of hits (n_{Tu}) as part of the total number of points was multiplied by the corresponding testis weight (W_t) to give an estimate (W_{Tu}) of seminiferous tubule weight (in grams) $W_{Tu} = \frac{n_{Tu}}{972} \times W_t$ It is assumed in this calculation that the specific weights of individual tissue components in the testis including the content of the tubular lumen, are identical The sum of W_{Tu} for the right and the left testis was taken as the tubular weight in each case

Clinical Data

Clinical data were recorded from the clinical notes and prepared for computer analysis

Statistical Analysis

Modified Student s t tests accounting for unequal variances and numbers of individuals were used for testing differences between arithmetic means and for testing differences between slopes of regression lines (*Snedecor & Cochran* 1967) n_A and n_B being the number of observations in the groups to be compared, p values were based on the least of $n_A - 1$ and $n_B - 1$ (for means) and $n_A - 2$ and $n_B - 2$ (for slopes) degrees of freedom P values below 0 05 were regarded statistically significant

Adjustment for age differences was performed according to the indirect method of standardization (*Armitage* 1971), using the age specific mean tubular weights of the main material of 172 patients as standard weights

Multiple regression analysis Stepwise and full multiple regression analysis was applied as previously described (*Haugen & Harbitz* 1972, *Harbitz* 1973 a), using tubular weight (X_1) as the *dependent* variable The following factors, either bivariate (1,0, labelled X_2 to X_{12}) or continuous (labelled X_{13} to X_{15}), were treated as *explanatory* (independent) variables

TABLE 1 *Histology of the Prostate by Age in 172 Patients*

Age	N	DA	BNH	C+BNH	C	AGP+BNH	AGP
40–49	1	1	2	0	0	0	0
50–59	11	5	10	3	1	5	0
60–69	7	1	22	15	5	6	1
70–79	0	1	28	21	0	5	1
80 +	0	0	9	9	0	2	0
Total	19	8	71	48	6	18	2

N = normal histology, DA = diffuse atrophy, BNH = benign nodular hyperplasia, C = carcinoma, AGP = atypical glandular proliferation

Histology of the Prostate

X_2 Benign nodular hyperplasia (BNH)
X_3 Atypical glandular proliferation (AGP)
X_4 Carcinoma (C)
X_5 Diffuse atrophy (DA)

Cause of Death

X_6 Cardiovascular disease*
X_7 Malignant tumour

Duration of Final Illness

X_8 *1–7 days*
X_9 >7 days

Other

X_{10} Steroid hormone treatment§
X_{11} Diabetes mellitus
X_{12} Liver cirrhosis
X_{13} Age
X_{14} Body weight
X_{15} Body length

Initially, forward stepwise regression analysis was run until all explanatory variables which were partially significant at the 5 per cent level at each step, were included Thereafter, the selected variables, together with all groups of prostatic histology (X_2–X_5), were included in the full multiple regression analysis Regression coefficients were calculated according to the method of least squares Differences between regression coefficients for the various groups of prostatic histology were tested by an F test (*Scheffé 1959*)

The analysis was based on a standard program for multiple regression (NRSR) developed at The Norwegian Computing Center, Oslo, and was conducted on a Univac 1108 computer

Reproducibility The reproducibility of the method for measuring the weight of seminiferous tubules was tested by duplicate determinations in 11 random cases The sections from both testes in these cases were recounted blindly the individual tubular weights were calculated, and the pairs of weights were compared (Fig 1) The method error, or the standard deviation of a single measurement, was 0 80, as computed from the usual formula $\sqrt{\Sigma D^2/2n}$, where D denotes the difference between duplicates and n the number of pairs of weights compared The method error was fair (5 9 per cent of the total mean tubular weight), although the number of duplicate measurements was small

* Includes death from myocardial infarction (49 cases), cerebrovascular and peripheral vascular disease (16 + 8 cases), rheumatic valvular disease (4 cases), miscellaneous cardiovascular disorders (11 cases)

§ Other than oestrogenic hormones Includes treatment with corticosteroids (7 cases), anabolic steroids (nortestosterone) (5 cases), or both (11 cases)

RESULTS

Fig 2 presents the frequency distribution of tubular weights in the set of 172 patients suitable for further analysis A slight positive skewness from the normal distribution was noted, but the median weight (13 20 grams) was nearly identical to the mean for the whole series (13 47 grams)

The scatter of individual observations of tubular weight was wide at all ages (Fig 3a), ranging from 0 01 to 31 49 grams Four cases in the 40–49 years age group all had relatively low tubular weights It seems unlikely that these observations, differing so markedly from those in higher decades, should be representative for men ageing 40–49 years All of them

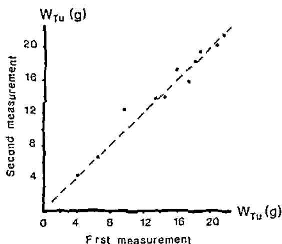


Fig 1 Reproducibility of tubular weight (W_{Tu}) determination in 11 cases examined on two occasions

had tubular weights which, on the average, were considerably lower than the mean weight for the whole series and the mean for those who died from cardiovascular diseases ($p < 0.001$) or other causes ($p < 0.01$) (Table 3) The standard deviations were high, and the means were altered only negligibly after adjustment for age differences between the groups If the final illness lasted for more than 7 days as defined here, tubular weight apparently declined and was markedly lower than in those who died shortly (< 1 day) or within 7 days after onset of symptoms ($p < 0.001$ and $p < 0.005$ respectively) Again, age adjustment caused no radical alteration in the means Patients who had had protracted duration of the terminal illness, and the three cases with the lower tubular weights had low testis weights and atrophic changes of one or both testes on microscopical examination

A slight decline in tubular weights with advancing age was observed, but the correlation coefficient (r) for the weight age relationship was low (–0 113) and statistically not significant The age specific mean weight for patients over 80 years of age was lower than the means in the younger age groups (apart from the fifth decade) (Table 2) but the difference was never statistically significant ($p > 0.10$)

Patients dying from malignant tumours

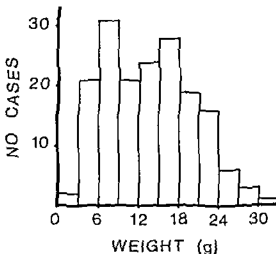


Fig 2 Distribution of the weight of seminiferous tubules at autopsy in the main series of 172 men aged 40 years or more

TABLE 2 *Weight of Seminiferous Tubules (Grams) in Men Previously not Subjected to Prostatic Surgery or Treatment with Oestrogenic Hormones*

Age	No patients	Tubular weight		
		Mean	S D	Range
40–49	4	6 54	2 12	4 15– 9 24
50–59	35	14 14	7 15	3 57 28 00
60–69	57	14 26	6 26	4 24 25 89
70–79	56	13 77	6 65	0 24–31 49
80 +	20	11 63	6 47	0 01–22 27
All	172	13 47	6 64	0 01 31 49

S D Standard deviation

TABLE 3 *Weight of Seminiferous Tubules (Grams) and Cause of Death, Duration of Final Illness, Steroid Hormone Treatment, Diabetes Mellitus and Liver Cirrhosis*

	No. patients	Tubular weight		
		Observed mean	S D	Age adjusted mean
Cause of death				
Cardiovascular disease	88	15 53	6 33	15 29
Malignant tumour	44	9 73	5 96	9 65
Other causes	40	13 56	6 17	13 63
	172			
Duration of final illness				
<1 day	37	17 23	4 87	16 66
1–7 days	35	16 52	6 54	16 36
>7 days	100	11 22	6 22	11 23
	172			
Other				
Steroid hormone treatment*	23	9 79	5 15	9 68
Diabetes mellitus	8	14 18	5 94	14 60
Liver cirrhosis	5	10 19	8 10	9 72
All	172	13 47	6 64	

S D Standard deviation
* Other than oestrogenic hormones.

TABLE 4 *Mean Weight of Seminiferous Tubules (Grams)* by Histology of the Prostate* and Age*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	(5 95)	(4 15)	8 02	–	–	–	–
50–59	12 24	6 54	18 30	17 04	(7 46)	17 19	–
60–69	18 16	(7 03)	16 40	11 14	10 04	13 63	(18 73)
70–79	–	(7 95)	13 27	14 70		13 20	(16 93)
80 +	–	–	8 65	13 73		15 59	–
All	14 09	6 48	14 22	13 55	9 61	14 71	17 83
S D	5 92	2 22	7 53	5 75	3 08	6 20	1 27

* For abbreviations and number of patients see Table 1
Figures in brackets refer to single observations
S D Standard deviation.

been treated with steroid hormones other than oestrogens or who had liver cirrhosis also showed mean tubular weights which were considerably lower than the mean for the whole series, but the standard deviations were high The mean tubular weight for the eight patients who suffered from diabetes mellitus was close to the total mean

The age specific and total mean tubular weights in relation to the histology of the

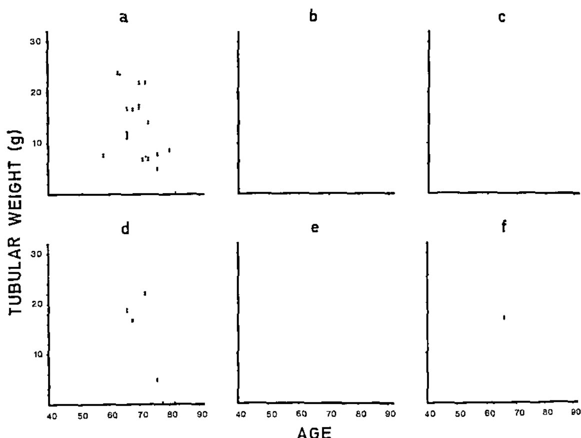


Fig 3 Tubular weight plotted against age in the main series of 172 men over 40 years of age (a) and in subgroups of cases with normal histology (b) diffuse atrophy (c) benign nodular hyperplasia only (d), carcinoma with (●) or without (○) benign nodular hyperplasia (e) and atypical glandular proliferation with (●) or without (○) benign nodular hyperplasia (f) of the prostate

prostate are presented in Table 4 In patients with normal prostatic histology tubular weight apparently increased from the sixth to the seventh decade, but the two means did not differ significantly from each other ($0.05 < p < 0.10$) On the other hand in patients with benign hyperplasia only (BNH) tubular weight seemed to decrease with advancing age Thus the mean weight in men over 80 years of age with BNH was considerably lower than that in the 50–59 years age group ($p < 0.02$) Among men over 80 years of age, those with benign hyperplasia only also had lower mean weight than those who had either carcinoma or atypical glandular proliferation and benign hyperplasia (C + BNH AGP + BNH) of the prostate However, the differences between the means were not statistically significant ($p > 0.10$ and $p > 0.20$ respectively)

Considering the mean tubular weights in patients with prostatic carcinoma or atypical glandular proliferation, no clear association with age appeared In the seventh decade, mean tubular weights in subjects with prostatic carcinoma either with benign hyperplasia (C + BNH) or without (C) were markedly lower than that in men with benign hyperplasia only ($p < 0.02$ and $p < 0.05$ respectively)

Patients with atypical glandular proliferation only (AGP) were few If, for reasons of comparison patients in the 60–69 and 70–79 years age groups are pooled those with AGP had higher mean weight than any other comparable group and, in particular, considerably

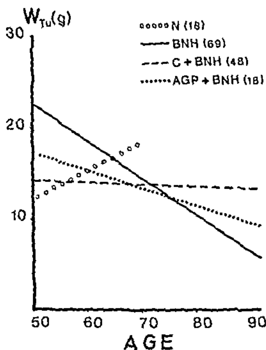


Fig 4 Regression of tubular weight (W_{Tu}) on age in men more than 50 years of age with benign nodular hyperplasia only (BNH), benign nodular hyperplasia and carcinoma (BNH + C), benign nodular hyperplasia and atypical glandular proliferation (BNH + AGP), and normal histology (N) of the prostate Figures in brackets indicate number of cases in each group

higher than the mean for patients with both atypical glandular proliferation and benign hyperplasia (*AGP + BNH*) However, the differences were never statistically significant (d f = 1) Finally, patients with diffuse *atrophy* of the prostate presented mean tubular weights which were constantly low and definitely under those for other histological groups at all ages

Scatter plots of individual tubular weight observations within histological groups are presented in Fig 3 The variation of weights was wide at practically all ages within all histological groups except that of diffuse atrophy of the prostate (Fig 3c), *in which* tubular weights were generally low and always under 10 grams The distribution of weights in the group of patients who had normal histology (Fig 3b) was apparently different from that in other groups at comparable age intervals However, the positive correlation ($r = 0.363$) between tubular weight and age in this group was not statistically significant ($p = 0.13$)

When patients over 50 years of age with prostatic hyperplasia only were considered (Fig 3d), a negative correlation between tubular weight and age was *demonstrable* ($r = -0.455$, $p < 0.001$), although the individual observations varied over a wide range at all ages On the other hand, no significant weight-age relationship was apparent in patients with both carcinoma and benign hyperplasia ($r = -0.042$) or in those with atypical glandular proliferation of the prostate ($r = -0.252$) The tubular weights among the few *patients who had prostatic carcinoma only* were generally low and seemingly increased with age ($r \approx 0.642$), but the correlation

TABLE 5 *Weight of Seminiferous Tubules (Grams) in Patients Previously Subjected to Prostatic Surgery**

Age	No patients	Tubular weight		
		Mean	S D	Range
50–59	3	8 69	2 37	6 95–11 39
60–69	5	12 24	7 89	6 44–25 11
70–79	7	10 44	4 85	4 61 17 97
80 +	9	10 96	6 32	4 27–21 24
All	24	10 79	5 69	4 27–25 11

* Two patients subjected to both prostatectomy and treatment with oestrogenic hormones not included
S D Standard deviation.

TABLE 6 *Weight of Seminiferous Tubules (Grams) in Patients who Received Oestrogenic Hormones (Diethylstilboestrol)*

Age	Duration of oestrogen treatment	Tubular weight	Testis weight*
69	1 month	2 75	15 5
70	1 ,,	4 71	18 6
68§	8 ,,	3 48	22 2
66§	12	0 00	1 9
76	4 years	0 00	14 9
79	4 ,,	0 00	10 7

§ Previously subjected to transvesical prostatectomy
* For details, see *Harbitz* (1973 a)

TABLE 7 *Relationship between Weight of Seminiferous Tubules and Various Explanatory Variables Expressed by Correlation Coeffecients Simple Correlation Analysis*

Explanatory variable	X_1 *Seminiferous tubule weight* (n_1 = 172)	
	Correlation coefficient	Significant at level
Histology of the prostate§		
X_2 BNH (− 137)	0 128	0 094
X_3 AGP (n = 20)	0 085	0 268
X_4 C (n = 54)	-0 037	0 629
X_5 DA (n = 8)	-0 233	0 002
Cause of death		
X_6 Cardiovascular disease (n − 88)	0 319	< 0 001
X_7 Malignant neoplasm (n − 44)	-0 331	< 0 001
Duration of final illness		
X_8 1–7 days (n − 35)	0 189	0 013
X_9 >7 days (n = 100)	0 402	< 0 001
Other		
X_{10} Steroid hormone treatment* (n − 23)	-0 219	0 004
X_{11} Diabetes mellitus (n = 8)	0 024	0 759
X_{12} Liver cirrhosis (n − 5)	-0 086	0 262
X_{13} Age (n − 172)	-0 113	0 140
X_{14} Body weight (n = 172)	0 313	< 0 001
X_{15} Body length (n − 172)	0 052	0 501

§ For abbreviations see Table 1
n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continuous variables)
n_1 Number of cases in which the weight of seminiferous tubules was recorded
* Other than oestrogenic hormones

coefficient was not statistically significant (0 05 < p < 0 10) at the chosen level of probability

Fig 4 shows the regression lines for tubular weight on age in patients over 50 years of age with normal histology of the prostate, benign nodular hyperplasia alone, and benign hyperplasia together with either carcinoma or atypical glandular proliferation The regression coefficient for the BNH group (b = -0 41) differed significantly (p < 0 01) from that for the C + BNH group (b = -0 028)

TABLE 8 *Weight of Seminiferous Tubules and Histology of the Prostate Full Regression Analysis*

Explanatory variable	Y_1 Seminiferous tubule weight (n_1 = 172) Partial correlation coefficient	Partial regression coefficient	Significant at level
Histology of the prostate§			
X_2 BNH (n = 137)	0 056	0 922	0 470
X_3 AGP (n = 20)	0 060	1 138	0 440
X_4 C (n = 54)	0 011	0 152	0 885
X_5 DA (n = 8)	-0 090	-2 890	0 248
Other			
X_9 >7 days (n = 100)	-0 257	-3 567	0 001
X_{14} Body weight (n = 172)	0 170	0 078	0 028
X_6 Cardiovascular disease (n = 88)	0 155	2 002	0 046
Multiple correlation coefficient (R)	0 490		< 0 001

§ For abbreviations, see Table 1
n and n_1 For explanation, see Table 7

whereas the difference from the regression coefficient for the AGP + BNH group (b = -0 195) was not statistically significant (p > 0 20) The difference between the slopes for the two lines for the C + BNH and AGP + BNH groups was far from the level of significance The positive association between tubular weight and age in cases with normal prostatic histology contrasted the negative slopes of the regression lines for the other histological groups

The age specific mean seminiferous tubule weights in patients who had been subjected to prostatic surgery for benign hyperplasia of the prostate were generally lower than those in non operated cases (Tables 2 and 5) but the standard deviations were considerable Nevertheless the total mean for the former was significantly lower than mean tubular weight for the 172 non operated cases (p < 0 05)

The weight of seminiferous tubules in the six patients who had been treated with oestrogenic hormones was generally markedly reduced In all cases, tubular weight constituted a relatively small portion of the testis weight The patients who had received oestrogens for 12 months or more had tubular weights at zero which implies that their tubules were shrunken and completely sclerotic (Table 6)

Individual simple correlation coefficients for the association between tubular weight and any explanatory variable to be included in the multiple regression analysis appears from Table 7 Tubular weight was positively correlated with body weight at a high significance level Among the bivariate variables tubular weight seemed positively correlated with cardiovascular disease (X_6) and short duration (1–7 days) of the terminal illness On the other hand, diffuse atrophy of the prostate (X_5) death from malignant neoplasm (X_7), protracted disease prior to death (X_9) and treatment with steroid hormones other than oestrogens (X_{10}) were all negatively correlated with tubular weight at the 5 per cent level of significance

Multiple Regression Analysis

At stepwise procedure of the multiple regression analysis, three regressors caused a significant reduction in the variance of tubular weight Long duration of the final illness (X_9) was selected at the first step and cardiovascular disease (X_6) and body weight (X_{14}) at the following steps their partial correlation coefficients being significant at the <0 1, 1 7 and 2 3 per cent levels respectively The

multiple correlation coefficient (R) for this set of regressors was 0 469 ($p < 0.001$), which means that they explained 22 0 per cent (R^2) of the variation in tubular weight in this series

If full multiple regression analysis was run with the regressors for prostatic histology (X_2–X_5) and the set selected at stepwise procedure (Table 8), the multiple correlation coefficient would be increased to 0 490 The significance levels for the individual regression coefficients of the histological groups were high The introduction of prostatic histology caused a negligible increase in the explanatory value of the regression (R^2 = 0 240), and the significance levels for the regression coefficients of X_8, X_9 and X_{14} were only moderately reduced The partial regression coefficients for individual groups of prostatic histology did not differ significantly from each other ($F = 1\,071$, $f_1 = 4$, $f_2 = 164$, $p > 0\,25$)

COMMENT

The morphology and function of the testis is under the control of pituitary hormones, and deficiency in pituitary gonadotropins results in progressive loss of germinal epithelium and shrinkage of the seminiferous tubules (*Turner* & *Bloodworth* 1968, *Steinberger* 1971) A correlation between the height of the germinative epithelium and the tubular width has been claimed, and tubular diameter is regarded as a significant indicator of testicular function (*Albert et al* 1953, *Johnsen* 1970)

Considerable inaccuracies are necessarily involved in the semiquantitative methods ordinarily used in the evaluation of the spermatogenetic epithelium Due to postmortem autolysis of the testes, such errors may even be strengthened when the maintenance of spermatogenesis is evaluated in autopsy material An absolute measure of the amount of tubular tissue, in this study based upon the application of point sampling technique and calculated as tubular weight was therefore considered preferable

There is no general agreement whether morphological changes occur in the human testis as the effect of ageing Many authors claim that the activity of spermatogenesis decreases, that tubular size is reduced and that the tunica propria of the seminiferous tubules thickens with increasing age, although these changes are not necessarily present in all cases and may vary within different parts of the testis (*Spangaro* 1902, *Sniffen* 1950, *Albert et al* 1953, *Burgi* & *Hedinger* 1959, *Sokal* 1964, *Suoranta* 1971) Although a slight reduction in age specific mean weights occurred, no significant alteration in tubular weight with advancing age could be demonstrated in the present investigation This probably reflects that the tubular diameter and the height of the spermatogenic epithelium is also generally unaltered in advanced years

The failure to observe a significant difference between partial regression coefficients for individual groups of prostatic histology at multiple regression analysis implies that tubular weight *per se* does not yield any diagnostic supplement in the prediction of neoplasia or hyperplasia of the prostate On the other hand, a negative correlation between tubular weight and age in patients with prostatic hyperplasia (BNH) was conspicuous and differed significantly from the corresponding relationship in patients with prostatic carcinoma (C) Compared with previous studies of prostatic weight in the same series of men (*Haugen* & *Harbitz* 1972), it appears that this declining amount of the seminiferous tubule component in advanced years in men with BNH is compatible with an increasing size of the hyperplastic prostate The prostatic carcinomas diagnosed in the present series were considered to represent early stages (*Harbitz* & *Haugen* 1972, *Haugen* & *Harbitz* 1972), and were apparently not associated with age-dependent alterations in the tubular mass

Among the regressors showing a statistically significant correlation with tubular weight at simple analysis, diffuse atrophy of the prostate, death from malignant tumours and treatment with steroid hormones other

than oestrogens were not selected at the multiple regression analysis This is probably due to interrelations of the three regressors with each other or with protracted disease before death, and body weight, which were both selected at significant levels at the multiple regression analysis

The negative correlation between tubular weight and long duration of illness was to be expected, since debilitating disease and improper nourishment are well known causes of impairment of testicular function and failure of spermatogenesis (*Hotchkiss* 1944, *Zubiran* & *Gomez Mont* 1953, *Leathem* 1961) The association between tubular weight and body weight probably also reflects only the dependence of tubular weight upon the nutritional state, since tubular weight showed no correlation with body length

The apparent relationship of tubular weight to cardiovascular disease is surprising Its clinical implications are difficult to explain, and the correlation may be an incidental circumstance in this series only On the other hand, it should not be overlooked that this regressor, at stepwise procedure, was selected second only to protracted disease (X_8) at a high level of significance ($p < 0.001$ at step 2), which might imply a real association between tubular weight and cardiovascular disease *per se*

The observation of lowered weight of seminiferous tubules in men who had been subjected to prostatic surgery is surprising and may be due to chance only It is unlikely that a damage to the ejaculatory ducts after prostatectomy (*Nilsson et al* 1969) should result in an atrophy of the tubules (cfr *Tillinger* 1957) The maintenance of the seminiferous tubules is probably closely related to the hormonal stimulation by Leydig cells (*Turner* & *Bloodworth* 1968) and Sertoli cells (*Lacy* & *Pettitt* 1970), and the quantity of these components remained apparently unaltered after prostatectomy (*Harbitz* 1973 b, c)

The observations in the few patients who received diethylstilboestrol for prostatic carcinoma confirmed previous reports on suppression of spermatogenesis and severe degeneration of the seminiferous tubules following treatment with oestrogenic hormones (*de la Balze et al* 1954, *Nylander et al* 1967, *Steinberger* 1971)

Hypospermatogenesis and disorders of the sexual function are frequently observed in experimental animals and men with diabetes mellitus (*Schöffling et al* 1963, 1967, *Federlin et al* 1965, *Faerman et al* 1972), and the magnitude of testicular changes varies with the severity of the diabetic condition (*Schöffling et al* 1967) In the present series, tubular weights in the patients with diabetes mellitus were apparently not altered by the metabolic disorder, which was generally of mild type Although the small number of cases do not allow any conclusions, the observations suggested that the amount of tubular tissue is maintained in elderly men with a mild course of diabetes mellitus

The findings presented in this report give further information about the maintenance of the seminiferous tubules in elderly men Apparently, benign prostatic hyperplasia occurred in men whose tubular function was declining in advanced years, whereas early carcinoma of the prostate was associated, to a larger extent, with preserved tubular mass throughout old age This may reflect differences in the hormonal environment which are biologically related to the development of benign hyperplasia and carcinoma of the prostate The lack of accurate knowledge concerning the endocrine control of spermatogenesis makes it difficult to interpret the morphological variations observed in the direction of specific hormonal disorders However, the importance of androgenic hormones in the control of spermatogenesis is repeatedly stressed and the present results are in accordance with the assumption that the balance between androgenic and oestrogenic hormones may be gradually displaced in favour of oestrogens in men developing prostatic hyperplasia

It seems well documented that, in addition to androgenic hormones, the gonadotrophic hormones of the adenohypophysis are of im-

portance for the morphological and physiological aspects of spermatogenesis, although the knowledge of the interactions between the gonads and the pituitary gonadotrophins is limited (*Steinberger* 1971) Stimulatory effects of ICSH (interstitial cell stimulating hormone) and FSH (follicle stimulating hormone) on the seminiferous tubules have been demonstrated in experimental animals and man (*Mancini et al* 1971, *Odell* & *Moyer* 1971, *Steinberger* 1971), although it has not been settled whether the effects are direct, indirect or a combination

In view of these reports, morphological variations of the seminiferous tubules may be caused by altered pituitary gonadotrophin activity as well as by alterations in the influence from androgenic and oestrogenic hormones *Haugen* (1973) observed, in the same series of men presented here, higher proportions of PAS positive (gonadotrophin-producing) cells of the adenohypophysis in men with hyperplasia and neoplasia of the prostate than among controls, which might suggest that the pituitary function was abnormal in these patients The present observations on tubular weight may also imply, in addition to derangement in the sex hormone balance, that it is the pituitary function which is disturbed in men with benign hyperplasia of the prostate The lack of a significant increase in the proportion of PAS positive cells in the hypophysis with age (*Haugen* 1973) in these patients could indicate a failing ability of the adenohypophysis to respond to a primary reduction in the seminiferous tubule component Similar in direct indications of disturbed pituitary function in patients with prostatic carcinoma were not demonstrable, although it has previously been reported that the urinary excretion of gonadotrophic hormones is increased and that the pituitary reserve of luteinizing hormone (= ICSH) is elevated in these patients (*Stern et al* 1964, *Geller et al* 1970)

Actuary *Ingar Holme*, Cand real, The Norwegian Computing Center Oslo Norway performed the computer analysis and gave valuable advice

The technical assistance of Mrs *B Eriksen* and Mrs *E Jareg* is gratefully acknowledged

Part of this study was performed while the author was a research fellow under *The Norwegian Society for Fighting Cancer* and *The University of Oslo* Financial support was thankfully received from *Direktor Gotfred Lie og hustru Marie Lies fond* and *Professor dr med Carl Sembs fond til medisinsk vitenskapelig forskning*

REFERENCES

1 *Albert, A, Underdahl, L O, Greene, L F* & *Lorenz, N* Male hypogonadism I The normal testis Proc Mayo Clin *28* 409–422, 1953
2 *Armitage, P* Statistical methods in medical research Blackwell Scientific Publications Oxford and Edinburgh 1971 504 pp
3 *Bacon, R L* & *Kirkman, H* The response of the testis of the hamster to chronic treatment with different estrogens Endocrinology *57* 255 271, 1955
4 *Balze, F A de la, Mancini, R E, Bur, G E* & *Irazu, J* Morphologic and histochemical changes produced by estrogens on adult human testes Fertility & Sterility *5* 421–436 1954
5 *Boccabella, A V* Reinitiation and restoration of spermatogenesis with testosterone propionate and other hormones after a long term post hypophysectomy regression period Endocrinology *72* 787–798, 1963
6 *Burgi, H* & *Hedinger, C* Histologische Hodenveranderungen im hohen Alter Schweiz med Wschr *89* 1236–1239, 1959
7 *Faerman, I, Vilar, O, Rivarola, M A, Rosner, J M, Jadzinsky, M N, Fox, D, Lloret, A P, Bernstein Hahn, L* & *Saraceni, D* Impotence and diabetes Studies of androgenic function in diabetic impotent males Diabetes *21* 23–30, 1972
8 *Federlin K, Schoffling, K, Neubronner, P* & *Pfeiffer, E F* Histometrische Untersuchungen am Hodengewebe des Diabetikers mit Keimdrusenunterfunktion Diabetologia *1* 85–90, 1965
9 *Geller, J, Baron, A* & *Kleinman, S* Pituitary luteinizing hormone reserve in elderly men with prostatic disease J Endocr *48* 289 290, 1970
10 *Harbitz, T B* Testis weight and the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *81* 148–158 1973 a
11 *Harbitz, T B* Morphometric studies of the Leydig cells in elderly men with special reference to the histology of the prostate An ana

lysis in an autopsy series Acta path microbiol scand Section A *81* 301–314, 1973 b
12. *Harbitz, T B* Morphometric studies of the Sertoli cells in elderly men with special reference to the histology of the prostate An analysis in an autopsy series Acta path microbiol scand Section A *81* 703–714, 1973 c
13. *Harbitz, T B* & *Haugen, O A* Histology of the prostate in elderly men. A study in an autopsy series. Acta path microbiol scand Section A *80* 756–768, 1972
14. *Haugen, O A* Distribution of pituitary cell types in relation to the histology of the prostate in elderly men An analysis in an autopsy series Acta path microbiol scand Section A *81* 411–424, 1973
15. *Haugen, O A* & *Harbitz, T B* Prostatic weight in elderly men An analysis in an autopsy series. Acta path microbiol scand Section A *80* 769–777, 1972
16. *Heller, C G, Nelson, W O, Hill, I B, Henderson, E, Maddock, W O, Jungck, E C, Paulsen, C A* & *Mortimore, G E* Improvement in spermatogenesis following depression of the human testis with testosterone Fertility & Sterility *1* 415–420, 1950
17. *Hotchkiss, R S* Fertility in men A clinical study of the causes, diagnosis, and treatment of impaired fertility in men J B Lippincott Company, Philadelphia 1944 p 14–34
18. *Johnsen, S G* Testicular biopsy score count — A method for registration of spermatogenesis in human testes Normal values and results in 335 hypogonadal males Hormones *1* 1–24, 1970
19. *Lacy, D* & *Pettitt, A J* Sites of hormone production in the mammalian testis, and their significance in the control of male fertility Brit med Bull *26* 87–91, 1970
20. *Leathem, J H* Nutritional effects on endocrine secretions In Young, W C (Ed) Sex and Internal Secretions 3 ed vol 1 The Williams & Wilkins Co, Baltimore 1961 p 666–704
21. *Ludwig, D J* The effect of androgen on spermatogenesis Endocrinology *46* 453–481, 1950
22. *Mancini, R E, Vilar, O, Donini, P* & *Lloret, A P* Effect of human urinary FSH and LH on the recovery of spermatogenesis in hypophysectomized patients J clin Endocr *33* 888–895, 1971
23. *Nelson, W O* & *Merckel, C* Maintenance of spermatogenesis in testis of the hypophysectomized rat with sterol derivatives Proc Soc exp Biol *36* 825–828, 1937
24. *Nilsson, S, Hultén, L* & *Seeman, T* State of the ejaculatory ducts, deferent duct ampullae, seminal vesicles and posterior urethra after various operations for benign prostatic hyperplasia A vasovesiculographic and urethroscopic study Scand J Urol Nephrol *3* 73–78, 1969
25. *Nylander, G, Persson, B H* & *Terner, N* Testis' morfologi vid ostrogen terapi Nord Med *78* 1493–1494, 1967
26. *Odell, W O* & *Moyer, D L* Physiology of reproduction The C V Mosby Company, Saint Louis 1971 p 82–94
27. *Prader, A* Testicular size Assessment and clinical importance Triangle *7* 240–243, 1966
28. *Roosen Runge, E C* Quantitative investigations on human testicular biopsies. I Normal testis Fertility & Sterility *7* 251–261, 1956
29. *Scheffé, H* The analysis of variance John Wiley & Sons, Inc, New York 1959 p 25 51
30. *Schoffling, K, Federlin, K, Ditschuneit, H* & *Pfeiffer, E F* Disorders of sexual function in male diabetics Diabetes *12* 519–527, 1963
31. *Schoffling, K, Federlin, K, Schmitt, W* & *Pfeiffer, E F* Histometric investigations on the testicular tissue of rats with alloxan diabetes and Chinese hamsters with spontaneous diabetes Acta endocr (Kbh) *54* 335–346, 1967
32. *Snedecor, G W* & *Cochran, W G* Statistical methods 6 ed The Iowa State University Press, Ames, Iowa 1967 593 pp
33. *Sniffen, R C* The testis I The normal testis Arch Path *50* 259 284, 1950
34. *Sniffen, R C, Howard, R P* & *Simmons, F A* The testis IV Idiopathic eunuchoidism with low FSH, Testicular changes secondary to lesions in or near the pituitary and secondary to estrogen therapy Arch Path *57* 464–480, 1954
35. *Sokal, Z* Morphology of the human testes in various periods of life Folia morph *23* 102–111, 1964
36. *Spangaro, S* Über die histologischen Veranderungen des Hodens, Nebenhodens und Samenleiters von Geburt an bis zum Greisenalter Anat Hefte *18* 593–771, 1902
37. *Steinberger, E* Hormonal control of mammalian spermatogenesis Physiol Rev *51* 1 22 1971
38. *Stern, E, Hopkins, C E, Weiner, J M* & *Marmorston, J* Hormone excretion patterns in breast and prostate cancer are abnormal Science *145* 716–719, 1964
39. *Suoranta, H* Changes in the small blood vessels of the adult human testis in relation to age and to some pathological conditions. Virchows Arch Abt. A Path Anat *352* 165–181, 1971
40. *Tillinger, K G* Some histological findings in testicles with blocked excretory ducts Acta path microbiol scand *41* 213–218, 1957
41. *Turner, J H* & *Bloodworth, J M B, Jr*

The testis In Bloodworth, J M B, Jr Endocrine Pathology Williams & Wilkins, Baltimore 1968 p 430–477

42 *Walsh, E L, Cuyler, W K & McCullagh, D* The physiologic maintenance of the male sex glands The effect of androtin on hypophysectomized rats Amer J Physiol *107* 508 51?, 1934

43 *Zubiran S & Gomez Mont, F* Endocrine disturbances in chronic human malnutrition Vitam and Horm *11* 97–132, 1953

Acta path microbiol scand. Section A 81, 857-861, 1973

PROGRESSIVE OCCLUSIVE ENDARTERITIS

A Case Report

R. Harket, O. Fausa and K. Nygaard

Institute of Pathology, Medical Department A and Surgical Department B, Rikshospitalet, University Hospital, Oslo, Norway

A case of fatal arterial disease characterized by intimal proliferation with occlusion of small and medium sized arteries is reported. The pathological picture corresponded to that seen in *Köhlmeier-Degos'* cutaneo-systemic disease. Clinically the patient showed a multisymptomatic disease, ending fatally due to intestinal gangrene. The typical skin lesion described in *Köhlmeier-Degos'* disease was, however, not observed. Although differing in some respects from the classical features of this disease, the reported case is believed to be of a similar nature.

During the last decades cases of widespread occlusive endarteritis have been reported as "malignant atrophic papulosis" or *Köhlmeier-Degos'* disease (1, 3, 9, 11). The dermal vascular lesion has a characteristic appearance and is considered pathognomonic, but identical vascular changes may occur in any organ. Recently we have observed a case of generalized occlusive intimal fibrosis without typical skin lesions. This report describes the widespread arterial involvement and discusses the implications of *Köhlmeier-Degos'* disease as a systemic, rather than a primary cutaneous disease.

CASE REPORT

In 1964 a 20 year-old man developed generalized arthralgia, accompanied by chronic uveitis, non characteristic exanthema and asymptomatic aortic valve insufficiency. During the following years h[illegible] had exanthemata of short d[illegible] and modera[illegible] joint symptoms without obj[illegible] Ep[illegible] of abdominal pain and jaund[illegible] attribut[illegible] cholelithiasis. In Septem[illegible] 19[illegible] suffere[illegible] from an acute illness with abdomi[illegible] and sore throat. High[illegible] doses of [illegible]

Received 10 iii 73 [illegible]ted 13 [illegible] 73

Vibramycin had no effect and he was transferred to Rikshospitalet on October 16th, 1970.

Clinical findings. He was severely ill, emaciated, and showed mouth ulcerations, uveitis and pleomorphic exanthema. The abdomen was distended with a questionable right sided mass.

Laboratory findings. ESR 39 mm/h, Hb 12.8 g/100 ml (after 2 blood transfusions), R.B.C. 4.49 mill., W.B.C. 20 700, platelet count 421 000. Serum protein 5.6 g/100 ml. AST, Waaler's-, Latex- and LE-tests were normal. Chest x-ray showed bilateral infiltration and right sided pleural effusion.

Treatment and course. Large doses of penicillin and streptomycin were given. Prednisone treatment, 60 mg daily, was continued and azathioprine, 100 mg daily, was added. Initially some improvement was noted, but on October 29th he showed signs of peritonitis. Laparotomy disclosed extensive gangrene of the large and small bowel with perforations. The colon and most of the small intestine was re[illegible]ected.

Pos[illegible]opera[illegible]ively sepsis ensued in addition to bilateral [illegible]neumonia, pericarditis, and increasing ja[illegible]ndi[illegible]e. He died 50 days after the operation.

PATHOLOGICAL FINDINGS

[illegible]al specimen. The small and large intestine [illegible]ved several gangrenous and perforated areas [illegible]d the serosal surface was covered by fibrin.

Post mortem examination. The wall of the remaining bowel was oedematous, and small foci of



necrosis were seen in the mesentery The liver was congested and showed yellow mottling on the cut surface A rudimentary gall bladder without stones was found The spleen showed a small infarction while the kidneys were without gross changes Small, nodular thickenings were seen on the aortic valves Except for some atheromatous plaques in the abdominal aorta no gross abnormalities were found in the great arteries

Histology The most distinctive finding was a widespread obliterative intimal fibrosis mainly affecting medium sized and smaller arteries This lesion involved the gastrointestinal tract, liver, gall bladder, pancreas, spleen, adrenals, kidneys and heart The arteries showed a concentric prolifera tion of the intima, often in several layers, narrow ing the lumen to a varying degree The internal elastic lamina was generally preserved Occasion ally, reduplication of the lamina was found and in some arteries with marked intimal hyperplasia, interruption or complete loss of the elastic lamina was seen The media, although often reduced to a small peripheral rim, revealed no evidence of focal destruction or fibrinoid necrosis The associated veins were not involved (Figs 1–5)

In the mostly affected intestinal segments some arteries showed organizing thrombi, occasionally with recanalization In a single area, close to a necrosis, there was acute vasculitis and fibrin like material in the intima and in the arterial lumen Except for this, acute arteritis was never seen (Figs 6–7)

The aortic valves and the adjacent aortic intima and endocardium revealed small superficial fibrous plaques In the wall below there was fibrosis and patchy inflammatory infiltration Fungal micro-abscesses were seen in many organs. A focal glom erulonephritis was noted and fungi were demonstrated in the tufts. Skin and synovial membrane were normal

---

Fig 1 Artery from the perirectal tissue, showing intact internal elastic lamina and marked intimal proliferation Haematoxylin azophloxine saffron × 180

Figs 2 and 4 Branches of the left coronary artery and the superior mesenteric artery with atrophy of the media and concentric proliferation of the intima Note the uninvolved vein in Fig 4 v Gie son elastica × 40 (Fig 2) Haematoxylin azo phloxine saffron × 110 (Fig 4)

Fig 3 Small mesenteric artery showing interrup tion of the internal elastic lamina. v Gieson elastica × 60

Fig 5 Same artery as in Fig 3 Several layers of obliterative fibrosis of the intima are seen Haema toxylin azophloxine-saffron × 290



Fig 6 Occlusion of a mesenteric artery with irregular intimal proliferation Haematoxylin azophloxine saffron × 180

DISCUSSION

Clinically, the present case is characterized by a multisymptomatic disease, consisting of arthralgia, skin eruptions, uveitis, endocarditis and attacks of abdominal pain, ending fatally due to intestinal gangrene Histologically, the striking feature was an occlusive intimal fibrosis of medium sized and smaller arteries, sometimes with thrombosis and rarely with acute vasculitis

The clinical picture revealed some features of polyarteritis nodosa, but the arterial changes differed from those usually found in this disease The majority of the vessels showed conspicuous intimal changes, but no focal inflammatory destruction was found in the outer coats of the wall Arterial segments adjacent to a necrotic lesion in polyarteritis nodosa may display intimal proliferation only Necrotic lesions may have been over-



Fig 7 Acute vasculitis of a small mesenteric artery adjacent to a necrotic area Haematoxylin azophloxine saffron × 225

looked in our case, but even so, they seem to be too scanty to account for the widespread intimal changes

In rheumatoid arthritis, vascular involvement of many types have been described (2, 10) Proliferative endarteritis may develop in the digital arteries and occasionally in the visceral arteries as well Our patient did not present typical clinical or radiological features of rheumatoid arthritis, nor evidence of peripheral vascular disease

The relationship to *Buerger*'s disease is difficult to assess The disease is not well defined and scepticism concerning its identity is admitted (5 6, 12) Nevertheless, in cases of fatal abdominal crisis combined with thromboangiitis of the extremities, conspicuous changes are almost invariably found in the superior mesenteric artery (4, 8), contrasted to the findings in the present case

In 1941 *Köhlmeier* (7) reported a 21-year-old man with a characteristic papular skin eruption, combined with multiple intestinal perforations A year later a similar cutaneous intestinal disorder was published by *Degos et al* (3) They thought it to be a well defined entity. Since then several authors have reported cases of this disease (1) which has been named "malignant atrophic papulosis" It most often affects young males and usually runs a fatal course due to intestinal perforation The disease is characterized by a progressive intimal proliferation of medium sized and smaller arteries A fibrinoid necrosis in the intima and acute vasculitis may be found (9) By definition the skin is affected in all cases Variably, the vessels in the gastrointestinal tract, the brain and other organs may be involved (11)

In the present case, the vascular changes seem to correspond to those described in *Köhlmeier Degos'* disease, but in some respect the case differed from those previously described The aortic and endocardial lesions have not formerly been observed, and the skin eruption never had the appearance of malignant atrophic papulosis We feel, however, that the key lesion in *Köhlmeier-Degos'* disease is not the skin eruption, but the progressive arterial changes which give rise to a wide range of manifestations The skin lesion may represent only one facet of the basic vascular pathology Perhaps the emphasis should be placed on the one common feature progressive, occlusive endarteritis of unknown aetiology

REFERENCES

1 *Benson F & Bergman F* Kohlmeier Degos disease (malignant atrophic papulosis) Acta med Scand *184* 165 169 1968
2 *Bywater, E G L* Peripheral vascular obstruction in rheumatoid arthritis and its relationship to other vascular lesions Ann rheum Dis *16* 84 103, 1957
3 *Degos R, Delort, J & Tricot, R* Dermatite papulosquameuse atrophiante Bull Soc franc Derm Syph *49* 148–150, 1942
4 *Degos, R* Malignant atrophic papulosis a fatal cutaneo-intestinal syndrome Brit J Dermatol *66* 304–307 1954
5 *Fisher, C M* Cerebral thromboangiitis obliterans Medicine *36* 169 209, 1957
6 *Gore, I & Burrow, S* Reconsideration of

pathogenesıs of Buerger s dısease Am J Clın Path 29 319–330, 1958
7 *Kohlmeıer, W* Multiple Hautnekrosen beı Trombangııtıs oblıterans Arch Derm Syph (Berl) *181* 783–792, 1941
8 *Lausecker, H* Beıtrag zur ıntestınalen Form der Trombangııtıs oblıterans mıt Hauterscheınungen Acta dermat venereol *29* 369–387 1949
9 *Naylor, D , Mullıns, J F & Gılmore, J F* Papulosıs atrophıcans malıgna (Degos dısease) Report of fırst Unıted States case and revıew of lıterature Arch Derm *81* 189–197, 1960
10 *Scott, J T , Hourıhane D O , Doyle, F H , Steıner, R E , Laws J W , Dıxon, A St J & Bywaters, E G L* Dıgıtal arterıtıs ın rheumatoıd dısease Ann rheum Dıs *20* 224–233, 1961
11 *Strole, W E Jr , Clark, W H Jr & Isselbacher, K J* Progressıve, arterıal occlusıve dısease (Kohlmeıer Degos) A frequent fatal cutaneo systemıc dısorder New Engl J Med *276* 195–201, 1967
12 *Wessler, S , Mıng, S C Gurewıch, V & Freıman, D G* A crıtıcal evaluatıon of thromboangııtıs oblıterans New Engl J Med *262* 1149–1160, 1960

Acta path microbiol scand Section A *81*, 862–865, 1973

BRIEF REPORT

THE NATURE OF MINERAL DEPOSIT IN RAT COSTAL CARTILAGE

Anders Hjerpe, Bengt Engfeldt and Jan Erik Glas

Mineralization in costal cartilage of young growing rats occurs extracellularly in a central core The mineralized tissue was isolated by centrifuging a finely ground powder in acetone/bromoform mixture with a specific gravity of 1 78 g/cm^3 The mineralized powder has the X ray diffraction pattern of calcium hydroxyapatite with a molar Ca/P ratio of 1 62 or more These figures do not differ significantly from the theoretical value It is concluded that rat costal cartilage might serve as a suitable system for studies of the mineralization process Such studies are in progress

Chemical studies of the physiological process of mineralization in certain tissues encounter several problems The important biological task is to separate and isolate areas in well defined stages of mineralization Furthermore, there is the difficulty of obtaining enough material for analysis especially in the early stages of mineralization

A mineralizing tissue that could offer certain advantages in studies of this type is rat costal cartilage as described by *Alcoc & Reid* (1969) and *Smith et al* (1972) Mineralization of this tissue starts at a well defined age and body weight as a normal physiological process in young growing rats In guinea pig costal cartilage, a similar type of mineralization has been described by Thyberg et al The mineralization of costal cartilage of young experimental animals, however, seems to differ from that in the human being In their paper *Alcoc & Reid* suggested that rat costal cartilage might serve as a model for investigating the mineralization process However, Smith et al studied the mineral components during this mineralization and reported among other things a molar Ca/P ratio no higher than 1 2 From this finding they concluded that the system is of limited value from this particular viewpoint

Received 12 ix 73 Accepted 12 ix 73

The Department of Pathology II, Karolinska Institutet, Stockholm, Sweden

Requests for reprints should be addressed to Karolinska Institutet, Pathology Research Laboratories, Solnavagen 1, 104 01 Stockholm 60, Sweden

The aim of the present study is to elucidate this problem further in order to obtain more accurate information on the nature of the calcium deposits in costal cartilage and its relation to other physiological mineralizing systems

Methods

From pairs of Sprague Dawley rats 25, 50, 75 and 100 days of age the rib cartilage was dissected as described by *Alcoc & Reid*, separating the costal bone with a good margin Two ribs from each rat were fixed in methanol for 24 hours, then embedded in paraffin and sectioned at 5 micron Sections were either stained with alizarin to demonstate calcium (*Dahl* 1952) or exposed to ultra soft X-ray microradiography to study the density relationships The radiation was generated at 1 5 kV using an X ray tube with W-anode The resulting radiation had a wavelength of 8 10 Å (*Engstrom & Lindstrom* 1950)

The remaining costal cartilage was dried in several changes of acetone and ground to a particle size of smaller than 37 micron (≥400 mesh) using a Spex Freezer/Mill cooled with liquid nitrogen To the cartilage powders obtained in each age group an acetone/bromoform mixture with a specific gravity of 1 78 g/cm^3 was added and the suspension was centrifuged for one hour at 26 500 g, using a Sorvall RC2- B centrifuge equipped with a SE 12 rotor The supernatants were carefully sucked off and the pellets were washed three times in acetone and left to dry From these pellets, material was put in glass capillaries measuring about 0 3 mm in diameter for X ray diffraction analysis Diffraction patterns were recorded in a cylindrical Debye Scherrer camera with a diameter of 114 6 mm using Ni filtered Cu radiation at 36 kV and 18 mA To the rest of the powder, 200 µl 6 M HCl was added and the material was hydrolysed for 18 hours in sealed pyrex glass tubes The hydrolysates were diluted with 2 ml of water and from this diluted hydrolysate aliquots were taken for phosphate determination according to *Youngberg & Youngberg* (1930), amino nitrogen determination according to *Moore & Stein* (1948) and finally

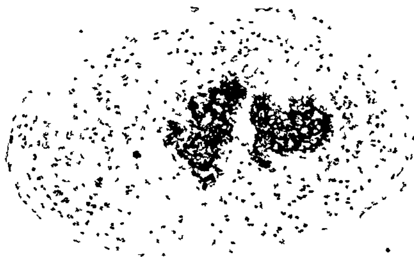


Fig 1 *Microphotograph of 5 micron thick paraffin embedded cross section of costal cartilage from a rat* 25 days of age Alizarin staining Magnification × 100

calcium determination by chelatometric titration with EGTA (Ethylene glycol bis-/2 aminoethyl ether/NNN N' tetraacetic acid) using a Marius calcium titrator Triple analyses were performed

Results and Comments

In transverse sections the alizarin staining (Fig 1) shows that calcium salts are mainly deposited in a central core of the cartilage and located extra cellularly In the case of rats 50 days and older this core covers approximately one third of the cross-section area while, in the case of the 25 day old rats this area was considerably smaller Ultra soft X ray analysis (Fig 2) also demonstrate that the matrix of this part of the cartilage has a considerably higher density clearly distinguishable from the non mineralized cartilage

The centrifugation yields and the results of chemical analysis are shown in Table 1 The X ray diffraction patterns (Fig 3) are characteristic of calcium hydroxyapatite and the diffuse reflexions indicate a fine crystalline structure similar to that found *in mineralized epiphyseal cartilage and bone*

The mineralization of the rat costal cartilage occurs only in the central part. This part has a higher density than the surrounding cartilage and can thus be isolated by centrifugation after careful grinding The yield figure shows a continuous increase from 25 to 100 days unlike the results given in earlier reports (*Alcoc & Reid* 1969 *Smith et al* 1972)

The tissue powder isolated in this manner has Ca/N and P/N ratios similar to those found in *dentine and bone in animals in the older age* groups while the 25 days group shows somewhat *lower values (Strandh 1960 Engfeldt & Hjerpe,* to be publ) This group also shows the lowest yield These low ratios could be due to a larger amount of composite particles because of the thin mineralized core in this group Another and more probable explanation supported by the microradiographic findings is that the particles are mineralized evenly but to a lower extent

The Ca/P ratio of the powder is close to the theoretical value of calcium hydroxyapatite 1 67 A lower value has been reported by Smith et al Their finding could be explained by the fact that they have analysed the whole costal cartilage including the nonmineralized portion This latter part with its relatively higher content of phosphate than of calcium constitutes the greater portion in *the early stages of mineralization*

Smith et al used atomic absorption for analysis of calcium In this procedure the Ca emission is depressed in the presence of phosphorus Too low

Fig 2a Microradiogram of 5 micron thick paraffin embedded cross-section of costal cartilage from a rat 75 days of age 1 5 kV The central mineralized area shows *a high X ray density Magnification* × 100

Fig 2b Microphotograph of the section used for fig 2a The section was transferred to tape and stained with haematoxylin eosin Magnification × *100*

Fig 2

TABLE 1. *The Recovery of Rat Costal Cartilage with Density Exceeding 1 78 g/cm³ in Rats of Different Age Groups*

Age in days	Amount of tissue ≥1 78 g/cm³ (per cent w/w)	Ca/N (w/w ratio)	P/N (w/w ratio)	Ca/P (w/w ratio)	Ca/P (molar ratio)
25	13	4 49	2 15	2 09	1 62
50	23	5 50	2 57	2 14	1 65
75	36	5 56	2 65	2 10	1 62
100	37	5 64	2 64	2 14	1 65

The degree and the character of mineralization are expressed as Ca/N and P/N ratios and as the Ca/P ratios



Fig 3 X ray diffraction pattern recorded from a powdered specimen of calcified costal cartilage from *a 75 days old rat showing the presence of apatite* which is poorly crystallized as indicated by the broadened diffuse lines

Ca-values are obtained if *phosphorus is not removed* before analysis or if adequate corrections are not included in the calculations Since no such corrections are mentioned in the paper it is unclear whether this circumstance has contributed to the low Ca/P ratio reported *by these authors*. However, such an error cannot explain the total divergence

Our results, including the crystallographic data, demonstrate that the mineral deposits are in the form of a calcium phosphate with characteristics of calcium hydroxyapatite The morphological findings together with the results of chemical analysis are thus similar to the findings obtained in the zone of calcification of the epiphyseal plate The mineralizing process in rat costal cartilage could thus be *expected to* represent a suitable model It has the additional advantage that the mineralized portion can easily be separated from its nonmineralized counterpart, either by microdissection or by centrifugation of finely ground material

This work has been supported by a grant from the *Swedish Medical Research Council*, project number K73-12x 540 09A and by funds from *Karolinska Institutet*, Stockholm, Sweden

References 1 *Alcoc, N W* & *Reid, J A* Biochem J *112* 511–513, 1969 —2 *Dahl, L K* Proc Soc Exp Biol *80* 474, 1952 —3 *Engfeldt, B* & *Hjerpe, A* To be published —4 *Engström, A* & *Lindstrom, B* Biochim biophys Acta *4* 351 373, 1950 —5 *Moore, S* & *Stein, W H* J Biol chem *176* 367–388, 1948 —6 *Smith, Q T*, *Olson D* & *Hamilton, M* Proc Soc Exptl Biol Med *140* 1096–1098 1972 —7 *Strandh, J* Experimental Cell Research *19* 515–530, 1960 —8 *Thyberg, J*, *Nilsson S* & *Friberg, U* Submitted Z Zellforsch mikrosk Anat —9 *Youngberg, G E* & *Youngberg M V* J Lab Clin Med *16* 158–166 1930

Acta path microbiol scand Section A *81*, 866-868, 1973

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting Stockholm, December 1, 1972

C Rubio & *B Lagerlof* AUTORADIOGRAPHIC STUDIES OF EXPERIMENTALLY INDUCED EPITHELIAL ATYPIAS IN THE UTERINE CERVIX

The cervical area in 93 C57B1 mice was painted twice a week with 1 per cent 3 4 benzpyrene in acetone for 1 to 5 months Following this procedure epithelial atypias of varying degrees developed in 72 of the 93 mice (77 per cent) Invasive carcinoma was also present in 17 mice Atypias were histologically classified on the basis of increased severity in Grade I, II and III

Autoradiographic studies "in vitro" with ^{3}H thymidin revealed increased nuclear labelling parallel to the degree of epithelial atypia Preliminary results indicated that the mean turnover rate in normal cervical epithelium is 36 hours, in moderate atypias (Grade II) 26 hours and in severe atypias (Grade III) 23 hours These results indicate a decreased generation time and turnover rate in the cervical epithelium presenting increasing degrees of mucosal alterations during carcinogenesis

R Willen CLASSIFICATION OF ORAL AND UPPER RESPIRATORY TRACT CARCINOMAS

In order to achieve a measurement of the biological activity of squamous cell carcinomas of the upper respiratory tract and oral cavity, a new system for the histological assessment of these carcinomas is proposed This system gives information of prognostic value better than that previously achieved from the primary biopsy

Method The analysis is based on the morphological picture of the tumour, the differentiation, cell polymorphism and mitosis frequency being the parameters Each receives points on a 1 to 4 scale Also the tumour host relationship is documented in terms of the mode and stage of invasion and grade of lymphocytic infiltration similarly scored

This method has been applied to a series of 124 cases of squamous cell carcinomas of the gingiva, to 110 cases with a similar lesion of the palate, and to 42 patients with squamous cell carcinomas of the larynx

Results An excellent correlation between the total scores of these tumours and the survival rates within the different groups has been found

In the group with lesions of the gingiva was also noted a correlation between total score and incidence of metastasis The finding of two peaks in the nomogram relating malignancy points, patient Nos and metastasis suggests the posibility that two types of squamous cell carcinomas may occur with differing biological activities in this region

Conclusion The above method of histological grading of these tumours appears to give a more exact measurement of the biological activity and thus the prognosis, than previously used systems It is also hoped that it will enable a more exacting choice of therapy for individual cases

S Cajander & *L Bjersing* A STUDY OF FOLLICLE RUPTURE IN THE RABBIT OVARY BY SCANNING ELECTRON MICROSCOPY

To elucidate the intra ovarian mechanism of follicle rupture, Dutch Belted rabbits were oophorectomized at accurately determined times before and after HCG-induced follicle rupture, ovarian changes, especially those on the surface of follicles, were studied mainly by scanning electron microscopy

Six hours after HCG injection the surface cells covering follicles were closer to each other than earlier and several displayed protruding vesicles. Such vesicles were not found between follicles Ten hours after injection, the surface cells at the base of the follicles had increased from about 8 to 14 μ in diameter and intracellular round bodies about 2 μ were visible Half way up to apex the surface cells were larger and thinner and contained several prominent intracellular bodies On the apex of the follicles the cells were collapsed and many were missing The underlying tissue was ischaemic

The structural changes were in accordance

Received 13 vii 73 Accepted 13 vii 73

with our working hypothesis regarding follicle rupture the extreme oestrogen and progestagen values in prerupture follicles and their vicinity cause labilization of lysosomes and local tissue degradation, collagen breakdown is initiated by apical ischaemia of these follicles and the consequential local decrease in the serum inhibitor of collagenase

T Moestrup & I Hagerstrand CANALICULAR ACTIVITY OF PHOSPHATASE IN HUMAN LIVER BIOPSY SPECIMENS—A PARAMALIGNANT MANIFESTATION?

In the normal human liver alkaline phosphatase activity is located to sinusoids In liver diseases the sinusoidal activity increases, and a faint canalicular activity may also be seen in cirrhosis, cholestasis chronic hepatitis and at the periphery of tumours Examination of 600 consecutive liver biopsy specimens stained by enzyme histochemical methods however, yielded 16 specimens showing a strong canalicular phosphatase activity in an otherwise morphologically normal liver tissue Malignant tumours were disclosed in 10 cases, 8 of which were autopsied Two clinically inapparent prostatic carcinomas were included Five of the 10 tumour cases had no malignant hepatic involvement — Malignancy is suspected in an additional 2 patients who are still alive Two cases of rheumatoid arthritis (1 with a mammary carcinoma), one case of temporal arteritis and 3 cases of alpha$_1$ antitrypsin deficiency (2 had simultaneous malignancy and showed diastase resistant MacManus positive globules of the hepatocytes) were among the 16 cases —In 13 cases a moderate elevation of serum alkaline phosphatase was found (below 25 Buch units) Isoenzyme determination yielded liver type enzyme in 5 cases examined In one case of renal carcinoma a previously raised serum value of alkaline phosphatase returned to normal after radical surgery

Goran Hansson, Lars Hamberger & Olov Nilsson AMINO ACID INCORPORATION *IN VITRO* INTO PROTEIN OF THE NORMAL HUMAN PARATHYROID GLAND AND THE PARATHYROID ADENOMA

The present investigation concerns the influence of calcium (Ca^{++}) on uptake and subsequent incorporation into protein of radioactively labelled leucin in normal parathyroid glands and in parathyroid adenomas

Slices of tissue from the parathyroid adenoma and one normal parathyroid gland taken from the same patient were incubated in the presence of ^3H leucin (0 01 mM) In the patients studied the incorporation of tritiated leucin into protein was higher in the normal parathyroid gland compared to the parathyroid adenoma when incubated for 30 and 60 min, respectively in TrisHCl buffer containing 1 5 mM Ca^{++} The protein synthesis in normal parathyroid glands was at a maximum when the calcium concentration in the medium was 1 5 mM and was significantly lower with 0 75 mM and 3 0 mM Ca^{++}, respectively However, corresponding alterations in the calcium concentration of the incubation medium produced no significant effects on the rate of protein synthesis in parathyroid adenomas suggesting that these behave autonomously in this respect

Studies are in progress concerning the influence of calcium (Ca^{++}) and magnesium (Mg^{++}) both on uptake of amino acids by use of the model amino acid α aminoisobutyric acid, and on the incorporation into protein of other normal amino acids

Lars Welin PERMEABILITY PROPERTIES OF MICROSOMAL MEMBRANES

Permeability studies of microsomal membranes are hampered by a lack of method, since the separation of particles from the surrounding medium is time consuming Millipore filtration is the only way to remove medium rapidly, but the interaction of substances with the filter itself invalidates the results Because of the slow sedimentation velocity of microsomal vesicles, a discontinuous gradient of perchloric acid—sucrose particle suspension cannot be employed We applied radioactivity and ultra centrifugation in the study of microsomal permeability Total water was estimated by using tritiated water, extraparticular water by dextran ^{14}C (MW 80,000) The adsorption of a number of substances prevented the investigation of several questions But the adsorption could be counteracted in many cases by using an excess of non labelled substrate Microsomal membranes are permeable to uncharged substances up to a molecular weight of at least 600, but they do not allow the penetration of any of the charged substances investigated, regardless of molecular weight. These findings could be further proved by analysis of osmotic properties since increasing concentrations of non permeable substances decrease the volume of the intramicrosomal water Microsomes suspended in distilled water do not show any damage, and the elevation of temperature when the microsomes are suspended in 0 1 M sucrose is also without effect On the other hand when microsomes are warmed up to 30° C in distilled water for 15 min followed by cooling in ice water bath the permeability increases to a great extent for charged substances As regards uncharged substances the situation remains unchanged, since inulin is still unable to reach the intramicrosomal compartment The permeability of mono- and divalent cations cannot be tested, since these substances adsorb to the highly negatively

charged surface of the microsomal membrane However, both substances induce strong osmotic response of total microsomes, which indicates that the majority of vesicles are impermeable even to small cations. In the initial phase of proteolytic and lipolytic enzyme action, no significant permeability increase could be observed, suggesting that the *permeability regulation is associated with deeper* hydrophobic regions

Olle Nilsson TRYPSIN SENSITIVITY OF MICROSOMAL MEMBRANES

Microsomal vesicles can be treated in a number of ways in order to remove membranous or membrane attached proteins. If microsomes are prepared in the absence of Mg^{++}, EDTA removes only bound ribosomes Alkaline buffer dissociates adsorbed protein, attached to the surface, which has a high net negative charged density Hypotonic treatment, together with warming and cooling, extracts the luminal proteins These proteins amount to 20 per cent, and the adsorbed protein makes up 30 per cent of the total microsomal protein Trypsin at maximal time and concentration solubilizes 20 per cent of the proteins localized on the outer surface *together with bound ribosomes Two enzymes,* NADPH cytochrome c reductase and cytochrome b_5, are also liberated In the presence of deoxycholate (DOC), 0 08 per cent per 4 mg protein in 1 ml, no protein is solubilized if alkaline washed and water treated microsomes were employed If non washed microsomes were treated with DOC, both adsorbed and luminal proteins were separated from the vesicles Permeability to ^{14}C labelled dextran of 80000 MW was analysed by the ultra centrifugation method After gradual increase of the permeability at 0 08 per cent final DOC concentration the intramicrosomal water volume was completely available to dextran. Since no phospholipids were removed by this procedure, one may conclude that channels are established through the membranes and large molecules can enter the luminal compartment This finding was used to study the transfer of trypsin into the lumen and, upon incubation the enzyme liberated 20 per cent of the microsomal protein which apparently is localized to the inner surface This procedure was used to analyse enzyme localization within the microsomal membranes DOC treatment is reversible since removal of the detergent by ultra centrifugation prevents dextran from entering the intramicrosomal water compartment In this way, various charged substrates could be included in the vesicle compartment and it became possible to study the action of various microsomal enzymes on substrates distributed along the inner surface

Gustaf Neander LIPID CARRIERS IN PANCREAS CYTOPLASMIC MEMBRANES

A number of enzymes produced by the endoplasmic reticulum and transported out from the exocrine pancreas cell to the digestive system are glycoproteins The intraluminal protein must be completed with oligosaccharide units within the channel, which means that the activated and water soluble sugar present in the cytoplasm has to pass the membrane barrier Recently, a lipid carrier, dolichol monophosphate (DMP), has been identified in liver microsomes which together with the participation of specific transferases mediates between the substrate and the product In this investigation, the possible presence and role of DMP was studied in membrane fractions isolated from guinea pig pancreas DMP from lipid extract was hydrolysed and purified on a DEAE cellulose column and the acceptor capacity of the isolated carrier was estimated by using excess of substrate and enzyme prepared from liver A high amount of DMP could be obtained both from rough microsomes and from Golgi membranes, approximately as much as in liver microsomes Using an external enzyme source DMP both from endoplasmic and Golgi membranes accept glucose, mannose, and N *acetylglucoseamine when the appropriate nucleo*tide derivatives are used as substrate As regards the liver, there is at present no evidence for the existence of different types of DMP in the various intracellular membranes In the case of pancreas, it is not yet possible to decide whether sugar acceptor specificity in microsomal and Golgi membranes contributes to sequential completion of the oligosaccharide chain. The specificity may be explained by the presence of individual transferases, non identical in the different fractions, and this was also analysed In the presence of substrate and purified liver DMP in excess subfractions from pancreas were used as transferase enzymes Endoplasmic membranes possess high activity for the transfer of sugar moiety from UDP glucose and GDP mannose to DMP, which property only in part is shared by Golgi membranes The Golgi fraction also contains UDP glucose transferase, but the enzyme for mannose transfer seems to be completely absent This finding fits in well with the sequential synthesis of export glycoproteins during the transport process, since the mannose moiety of isolated pancreas enzymes is located close to the peptide residue i e in a non terminal position It appears that dolichol monophosphate is present in different types of cytoplasmic membranes of the pancreas exocrine cell and contributes to the regulation of sugar transport to the intravesicular compartment where the prosthetic groups of the digestive enzymes are added to the ready polypeptide chain